MANIPULATION OF APOPTOSIS IN CANCER CELLS

Thesis submitted in accordance with the requirements
of the University of Chester
for the degree of Doctor of Philosophy by

Muneera Mohamed Sahib

February 2018

University of Chester

Declaration

I hereby declare that the research work presented in this thesis is authentic and has not been submitted previously in support of any course or qualification.

Signed:

Date:

Acknowledgements

I am always grateful to God Almighty for blessings me with this golden opportunity to carry out my PhD research at the School of Medicine, University of Chester, UK.

I would like to take this occasion to express my sincere gratitude to everyone who supported me throughout my PhD journey.

Firstly, I would like to express my sincere gratitude to my supervisors Prof. John. H.H. Williams and Dr. Elyse Ireland for providing me this opportunity and for being always being there for me with their invaluable supervision and guidelines throughout the research. I must also thank Mark for his valuable suggestions and Jasmine Stanley Ahmed for providing all the help in the laboratory. I would also like to thank Kate Coxon for all the help she did and all my fellow PhD friends for their help and support at the institute.

I extend my thanks to my husband for encouraging me and meeting all my financial needs to reach this stage. My thanks to my mum is enormous for looking after my daughter in India for the 2 years of my PhD. A big thank you to my little princess Ameekka for being good to me at home. And a special thanks to all my relatives for their immense support throughout.

Last, but not the least, I would like to acknowledge all the members of the Abbeyfield Wrexham Society limited, all the friends and families in Wrexham, Gap personnel Wrexham, Clark services for their support and encouragement throughout my research study.

Abstract

Conventional cancer therapies can have severe side effects, so new strategies to limit these needs to be investigated. Several anticancer agents induce the expression of tumour suppressor gene p21 in colorectal cancer cell line HT-29. Interestingly, the stress protein HSPA1A is also often elevated in tumour cells and has an anti - apoptotic activity. The main aim of this study was to examine whether a two - pronged approach, overexpressing p21 (using genetic approach and inhibition of HSPA1A using pifithrin - μ would be effective in inducing apoptosis in tumour cells. Chitosan or BSA based delivery systems were evaluated for cytotoxicity, with the intension of using it for plasmid DNA based cell transfections in this study. The interaction of HSPA1A protein in combination treatments involving UV radiation and hyperthermia at 42℃ were also evaluated to perceive the various roles of HSPA1A in arresting colorectal cancer cells. Colorectal cancer cell lines HT-29 and leukaemia cancer cell lines U937 were used in the study. All experiments were performed with cancer cell lines maintained in culture medium devoid of antibiotics. Cell cytotoxicity were evaluated using MTS and PI assays. The rate of apoptosis was determined using annexin V and PI staining by flow cytometry. Chitosan or BSA based microparticles or microgels were observed for size determination or morphology using scanning electron microscopy. Full length human p21 inserted plasmid DNA was a gift from Mien - Chie Hung, Addgene, USA. HT- 29 cells were subjected to p21 plasmid DNA transfection effects. Cells were treated with pifithrin - μ (15μM) prior to gene transfection to address its combined effect with p21 plasmid DNA transfection. HSPA1A and p21 protein expression studies were analysed using FITC labelled antibodies by flow cytometer. Combination studies with HSPA1A inhibitor pifithrin - μ and UV reflected enhanced cytotoxicity compared with either of the treatments independently. Hyperthermia at 42℃ induced apoptosis by MTS assay, which was confirmed by flow cytometric analysis in both the cell lines tested. Considering the cytotoxicity reflected by the chitosan or BSA delivery systems in drug free states, the p21 plasmid DNA transfection was carried out using lipofectamine 2000. Both overexpression of p21 and inhibition of HSPA1A protein with pifithrin - μ enhanced the rate of apoptosis with statistical significance of (p-<0.0001****) compared to the respected controls. The data in this thesis suggests the inhibition of HSPA1A in combination with increased p21 would be a promising therapeutic strategy for the treatment of colorectal cancers.

Contents

Chapter 1

Introduction

1.1 Cancer statistics……19

1.2 Factors associated with cancer initiation and progression……20

1.2.1 Cancer and apoptosis…… 21

1.2.2 Genes associated in proceeding apoptosis…… 21

1.2.3 Genes associated in hampering apoptosis……23

1.2.4 Heat shock genes and apoptosis…….. 23

1.2.5 Interactive role of tumour suppressor gene p53 and p21 in cancer ……..26

1.2.6 Genes associated with p21 upregulation……28

1.2.7 Up regulatory status of p21 in chemotherapeutic induced cancer arrest……..29

1.3 Role of p21 in cell cycle regulation and HSPA1A ……29

1.4 Apoptosis and necrosis in eukaryotic cells……30

1.5 Cancer treatments and issues addressed……31

1.5.1 Surgery……31

1.5.2 Radiation therapy……32

1.5.3 Chemotherapy……32

1.5.4 Combination therapy……33

1.5.5 Hyperthermia treatment……34

1.5.6 Natural anti - cancer agents……35

1.6 Strategies in improving the therapeutic potential of cancer therapies……36

1.6.1 Intracellular delivery entities - nanoparticles and drug delivery……36

1.6.2 Carbon nanoparticles in clinical research……40

1.6.3 Chitosan nanoparticles- properties and applications……40

1.6.4 Bovine serum albumin (BSA) nanoparticles……42

1.6.5 Lipid nano particles and transfection……43

1.6.6 Gene therapy……43

1.7 Rationale of the study……44

1.8 Objectives and hypothesis of the thesis……45

Chapter 2

Materials and methods

2.1 Materials……48

2.2 Preparation of tissue culture medium, reagents and buffers

2.2.1 Tissue culture medium ……54

2.2.2 Reagents for cell - based assays……54

2.2.3 Preparation of chemotherapeutics……55

2.2.4 Reagents for chitosan and BSA based microgels or microparticles……55

2.2.5 Reagents for agarose gel electrophoresis……56

2.2.6 Preparation of reagents and microbiological growth media……56

2.3 Methods

2.3.1Tissue culturing……58

2.3.2 Frozen cell storage and recovery……58

2.3.3 Determination of cell viability by microscope……59

2.3.4 MTS cell viability assay……59

2.3.5 Propidium iodide assay……60

2.3.6 Determination of annexin v and PI by flow cytometer……61

2.3.7 Flow cytometric determination of HSPA1A and p21 protein……61

2.3.8 Preparation of chitosan microgels……63

2.3.9 Preparation of chitosan microparticles ……64

2.3.10 Preparation of bovine serum albumin microparticles……65

2.3.11 Sample preparation for scanning electron microscopy……66

2.3.12 Bacterial culturing and storage……66

2.3.13 Extraction of p21 plasmid DNA from bacterial cells……67

2.3.14 Sub cloning of the pMT5 vector DNA ……68

2.3.15 Restriction of p21 gene and purification of pMT5 vector DNA ……68

2.3.16 p21 plasmid DNA map ……69

2.3.17 Electrophoretic separation of pMT5 vector……..69
2.3.18 Extraction and purification of pMT5 vector from agarose gel……70
2.3.19 DNA repair, purification and ligation of the pMT5 vector DNA……..70
2.3.20 Transformation of pMT5 vector DNA using DH5 α chemical competent cells……72
2.3.21 Transfection of the p21 plasmid DNA and vector DNA onto HT-29 cell lines……..73
2.3.22 Statistical analysis……73

Chapter 3

Effect of antimycotic/antibiotic on colorectal cancer cell line HT-29

3.1 Introduction…….. 74
3.1.1 Aims and hypothesis……75
3.2 Methods……75
3.2.1 Cell seeding and experimental design……..75
3.2.2 Treatment with antimycotic/antibiotic……75
3.2.3 Measurements of cell viability……..76
3.2.4 Determination of HSPA1A protein expression……76
3.3 Results
3.3.1 Determination of apoptosis and necrosis……..77
3.3.2 Evaluation of HSPA1A expression profiles post antimycotic /antibiotic on HT-29 cancer cell lines ……77
3.4 Discussion……85

Chapter 4

Inhibition of HSPA1A in combined Therapies

4.1 Introduction……87
4.1.1 Aims and hypothesis……89

4.2 Methods

4.2.1 Cell seeding and experimental design……90

4.2.2 UV irradiation exposure……90

4.2.3 Hyperthermia……90

4.2.4 Treatment with pifithrin - μ and pifithrin chloride……90

4.2.5 Measurements of cell viability……90

4.2.6 Determination of hyperthermia induced HSPA1A proteins……91

4.3 Results

4.3.1 Effect of UV on U937 cell lines…… 92

4.3.2 Effect of pifithrin - μ and pifithrin chloride on U937 cell lines……94

4.3.3 Effect of pifithrin - μ (20μM) and (10μM) combined with low UV radiation on U937 cell line ……101

4.3.4 Effect of hyperthermia 42◦C on U937 lines……105

4.3.5 Determination of hyperthermia induced apoptosis by flow cytometry……105

4.3.6 Hyperthermia and HSPA1A protein in U937 cell lines……105

4.3.7 Effect of pifithrin - μ and pifithrin chloride in HT-29 colorectal cancer cell lines……110

4.3.8 Effect of pifithrin - μ combined with UV (4 s) exposure on HT-29 cell line……115

4.3.9 Effect of hyperthermia in HT-29 cancer cell lines……117

4.3.10 Determination of hyperthermia induced apoptosis by flowcytometry……119

4.4 Discussion……121

Chapter 5

Intracellular delivery systems of chitosan and BSA

5.1 Introduction……124

5.1.1 Aims and hypothesis……125

5.2 Methods

5.2.1 Cell culture and experimental design……126

5.2.2 Chitosan - β - glycerol phosphate microgels treatment……126

5.2.3 Chitosan - glutaraldehyde microparticle treatment……126

5.2.4 Treatment with BSA microparticles……126

5.2.5 Determination of cytotoxicity and microscopic observations……126

5.3 Results

5.3.1 Microscopic observation of Chitosan β - glycerol phosphate microgels and chitosan microparticles……127

5.3.2 Cytotoxicity of β - glycerol phosphate on HT-29 cell lines……130

5.3.3 Effect of 5 - fluorouracil encapsulated chitosan microgels on HT-29 cell lines……132

5.3.4 Effect of 5 fluorouracil encapsulated glutaraldehyde linked chitosan microparticles on HT-29 cell lines……132

5.3.5 Effect of BSA microparticles with 5 - fluorouracil on HT - 29 cell lines……138

5.4 Discussion……140

Chapter 6

Effect of p21 plasmid DNA transfection and HSPA1A inhibition in colorectal cancer cell lines HT-29

6.1 Introduction……142

6.1.1 Aims and hypothesis……143

6.2 Methods

6.2.1 Cell culture……144

6.2.2 p21 plasmid DNA isolation, extraction, purification, transformation and transfection144

6.2.3 Pifithrin - μ assisted inhibition of HSPA1A protein and p21 plasmid DNA transfection 145

6.2.4 Measurement of cell viability, apoptosis, necrosis and protein expression profiles……145

6.2.5 Observation of apoptosis and protein expression profiles ……..……..145

6.2.6 Experimental design of p21 - plasmid DNA - extraction, purification and transfection146

6.2.7 Sequencing of p21 plasmid cDNA by Eurofins laboratories, UK ……..147

6.3 Results

6.3.1 Effect of p21 plasmid cDNA transfection and pifithrin - μ mediated HSPA1A inhibition and p21 plasmid cDNA transfection in HT-29 colorectal cancer cell lines……..148

6.3.2 Evaluation of pifithrin - μ assisted inhibition of HSPA1A in HT-29 cell lines……..153

6.3.3 Determination of intracellular and cell surface levels of p21 protein resulted from p21 plasmid DNA transfection by flow cytometer……..155

6.4 Discussion……158

Chapter 7

7.1 Discussion and future research……160

Chapter 8

References………169

List of Figures

Fig 1.1 The different stages of cancer initiation and metastasis……..……18

Fig 1.2 The diagram illustrates the p53 directed impaired apoptotic pathway in cancer cells.22

Fig 1.3 The diagram illustrates the involvement of stress induced HSPA1A protein in preventing apoptosis……25

Fig 1.4 The tumour suppressor gene mediated DNA repair……..……..27

Fig 1.5 The eukaryotic cell death routes……..31

Fig 2.1 Preparation of chitosan - β - glycerol phosphate modified microgel systems……..64

Fig 2.2 Preparation of Chitosan - TPP -5 - fluorouracil microparticles……..65

Fig 2.3 p21 plasmid DNA #16240 map……69

Fig 3.3.1 Effect on cell viability by antimycotic/antibiotic on HT-29 cell lines……78

Fig 3.3.2 Flowcytometric data presentation of annexin V and PI staining in HT-29 cell lines exposed to antimycotic/ antibiotic……79

Fig 3.3.3 Effect on cell viability by antimycotic /antibiotic on HT-29 cell lines by annexin V and PI staining……80

Fig 3.3.4 Intracellular HSPA1A expression resulted from antimycotic/ antibiotic treatment in HT-29 cell lines……81

Fig 3.3.5 Graphical representation of intracellular HSPA1A expression resulted from antimycotic / antibiotic treatment in HT-29 cell line……82

Fig 3.3.6 Cell surface HSPA1A expression resulted from antimycotic/ antibiotic treatment in HT-29 cell lines……83

Fig 3.3.7 Graphical representation of surface HSPA1A expression resulted from antimycotic/ antibiotic treatment in HT-29 cell lines……84

Fig 4.3.1 Microscopic observation of U937 cell lines treated with UV radiation ……92

Fig 4.3.2 The effect on cell viability by UV exposure on U937 cell lines……93

Fig 4.3.3 Microscopic observation of U937 cell lines treated with pifithrin-μ and pifithrin chloride……95

Fig 4.3.4 Effect on cell viability by pifithrin - μ on U937 cell lines……96

Fig 4.3.5 Effect on cell viability by pifithrin - μ on U937 cell lines……97

Fig 4.3.6 Microscopic observation of U937 cell lines treated with pifithrin-μ and pifithrin chloride……..98
Fig 4.3.7 Effect on cell viability by pifithrin chloride on U937 cell lines……99
Fig 4.3.8 Effect on cell viability by pifithrin chloride on U937 cell lines……100
Fig 4.3.9 Microscopic observation of U937 cell lines treated with pifithrin - μ and UV……102
Fig 4.3.10 Effect on cell viability by pifithrin - μ(20μM) with low UV on U937 cell lines……..103
Fig 4.3.11 Effect on cell viability by pifithrin - μ(10μM) combined with UV on U937 cell lines104
Fig 4.3.12 Effect on cell viability by hyperthermia 42℃ on U937 cell lines……106
Fig 4.3.13 Flowcytometric data presentation of annexin V and PI staining in U937 cell lines exposed to 42 ℃ heat shock treatment……107
Fig 4.3.14 Data interpretation of annexin V and PI staining in U937 cell lines exposed to 42 ℃ heat shock treatment……108
Fig 4.3.15 Expression of cell surface HSPA1A in U937 leukaemia cell lines exposed to 42 ℃ heat shock treatment……..109
Fig 4.3.16 Effect on cell viability by pifithrin - μ on HT-29 cell lines for 24 h……111
Fig 4.3.17 Effect on cell viability by pifithrin - μ on HT-29 cell lines for 48 h……112
Fig 4.3.18 Effect on cell viability by pifithrin chloride on HT-29 cell lines for 24 h……113
Fig 4.3.19 Effect on cell viability by pifithrin chloride on HT-29 cell lines for (48 h)……114
Fig 4.3.20 Effect on cell viability by pifithrin - μ combined with UV (4s) exposure on HT-29 cell lines……116
Fig 4.3.21 Effect of heat shock exposure on HT-29 cell lines……118
Fig 4.3.22 Flowcytometric data presentation of annexin V and PI staining in HT-29 cell lines

exposed to 42 ℃ heat shock treatment……..119

Fig 4.3.23 Graphical determination of apoptosis by annexin V and PI staining in HT-29 cell lines exposed to 42℃ ……..120

Fig 5.3.1 Scanning electron microscopic observation of β - glycerol phosphate crosslinked chitosan microgels ……127

Fig 5.3.2 Scanning electron microscopic observation of glutaraldehyde crosslinked chitosan microparticles……129

Fig 5.3.3 Effect on cell viability by β - glycerol phosphate concentration in chitosan microgels on HT-29 cell lines……131

Fig 5.3.4 Effect on cell viability by chitosan - β - glycerol phosphate (10 %) - 5-flurouracil microgels on HT-29 cell lines……133

Fig 5.3.5 Effect on cell viability by chitosan - β - glycerol phosphate (5 %) - 5 - fluorouracil microgels on HT-29 cell lines……134

Fig 5.3.6 Effect on cell viability by chitosan - β - glycerol phosphate (1 %) - 5 - fluorouracil microgels on HT-29 cell lines……135

Fig 5.3.7 Effect on cell viability by chitosan - β - glycerol phosphate (0.5 %) - 5 - fluorouracil microgels on HT-29 cell lines……136

Fig 5.3.8 Effect on cell viability by 5 - fluorouracil (glutaraldehyde cross linked, PH-8) chitosan microparticles on HT-2 cell lines……137

Fig 5.3.9 Scanning electron microscopic observation of BSA protein microparticles……138

Fig 5.3.10 Effect on cell viability by BSA microparticles loaded 5 - fluorouracil on HT-29 cell lines……..139

Fig 6.2.6.1 Isolated colonies of *Escherichia coli* containing p21 plasmid cDNA……146

Fig 6.2.6.2 Identification of p21 inserted plasmid DNA by agarose gel-electrophoresis……..146

Fig 6.2.6.3 Restriction digest of p21 plasmid cDNA from PMT5 vector DNA ……..146

Fig 6.2.6.4 Transformation of the ligated PMT5 vector into DH5 α competent cells……146

Fig 6.3.1 Microscopic observation of HT-29 cell lines treated with p21 plasmid cDNA transfection……..149

Fig 6.3.2 Effect of p21-plasmid DNA transfection on HT-29 cell lines……..150

Fig 6.3.3 Flowcytometric data presentation of p21 plasmid DNA transfection by annexin V and PI staining in HT-29 cell lines……151

Fig 6.3.4 Effect of p21 plasmid DNA transfection determination by annexin V and PI staining……152

Fig 6.3.5 Intracellular inhibition of pifithrin - μ facilitated HSPA1A protein expression in HT-29 cancer cell lines……..154

Fig 6.3.6 Intracellular p21 protein expression in HT-29 cancer cell lines……156

Fig 6.3.7 p21 protein expression at cell surface in HT-29 cancer cell lines……157

Fig 7.1 Interference of HSPA1A in cancer cells……166

Fig 7.2 Interference of HSPA1A in p21 transfection……166

Abbreviations

Apaf1	Apoptotic protease activating factor 1
ATG5	Autophagy protein 5
BCL-2	B cell lymphoma 2
BSA	Bovine serum albumin
PUMA	p53 upregulated modulator of apoptosis
BID	BH3 interacting domain
Bax	BCL2 associated x protein
BAD	BCL 2 associated death
CNT	Carbon nanotubes
EMEM	Eagle's minimal essential medium
5-FU	5 - Fluorouracil
TAE	Tris acetate EDTA
TNF-α	Tumour necrosis factor-α
MCL-1	Myeloid leukaemia cell differentiation
MTS	(4,5-dimethylthiazol-2yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium
PES	Phenazine ethosulphate
BCL-XL	B cell lymphoma extra large
ATP	Adenosine triphosphate
HSP70	Heat shock protein 70
HSPA1A	Heat shock protein A1A
HSP60	Heat shock protein 60

HSP90	Heat shock protein 90
HSP110	Heat shock protein 110
HSP27	Heat shock protein 27
HSP 71	Heat shock protein 71
LC3	Light chain -3
NEF	Nucleotide exchange factors
TPR	Tetratricopeptide chains
DR4	Death receptor 4
DR5	Death receptor 5
DMSO	Dimethyl sulphoxide
DPBS	I Dulbecco's phosphate buffered saline Roswell Park Memorial Institute
HAT	Histone acetyltransferase
FBS	Fetal bovine serum
FITC	Fluorescein
SMG7	Suppressor with morphological defects in genitalia
TGF	Transforming growth factor
TPP	Sodium tri-polyphosphate
MIR 13a	Micro RNA 13a
MIR34a	Micro RNA 34a
MIR106	Micro RNA 106a
MIR708-5	Micro RNA 708a
MIR708-5	Micro RNA 708 5P
PCNA	Proliferating cell nuclear antigen

PI	Propidium iodide
RPMI	Roswell Park Memorial Institute
FBS	Fetal bovine serum
SPRY	Sprouty proteins
SCLC	Small cell lung cancer
SOC	Super optimal catabolite repression
NSCLC	Non-small cell lung cancer
Dp44mt	di - 2 pyridylketone - 4 - 4 - dimethyl - 3 - thiosemicarbazone
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
NEF	Nucleotide exchange factors
TPR	Tetratricopeptide chains
TEMTAC	Tunable endogenous mammalian target complementation
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

The eukaryotic systems are regulated by various genes which directly or indirectly participate in maintaining normal cell replication. Malfunctional status of these cell cycle regulatory genes results in uncontrolled cell proliferation, the state known as cancer. Cancer cells are stimulated to release certain unique factors called metalloproteinases which facilitate their active propagation by assimilation of surrounding tissue walls, paving into the circulatory stream, to fasten their “roots” throughout the host system (Rundhaug. 2003). Cancerous cells upon entering the host circulatory stream proliferate to the surrounding tissues and reaches a stage clinically termed as metastasis (Fig 1.1).

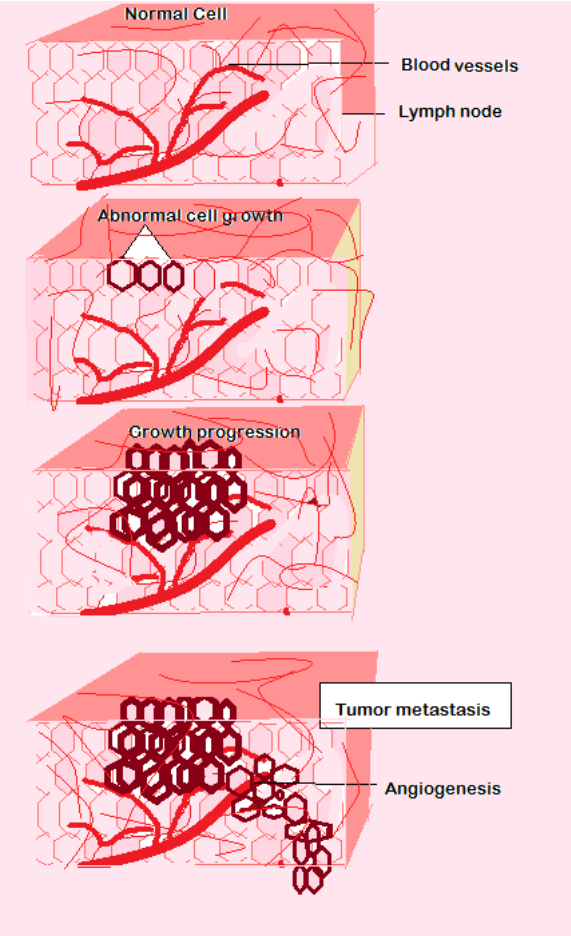


Fig 1.1 The different stages of cancer initiation and metastasis adapted from (drugresource.com; thetruthaboutcancer.com)

Metastatic phase of cancer leads to poor prognosis rate with conventional treatments. In case of pancreatic malignancies metalloproteinases are found to induce its activity at the primary cancer stage (Bloomston et al. 2002; Hanahan. 1996). Despite many researchers over half a century focusing on cancer vaccine innovations and dissecting the underlying molecular connections related to abnormal gene expressions, tumour angiogenesis, apoptosis and metastasis, cancer remain a leading cause of mortality compared to other disorders.

1.1 Cancer statistics

Data from worldwide cancer statistical analysis reveals the incidence of different types of cancers rising at an alarming rate. Publication record from a recent analysis had shown that in United states, in women, breast cancer is the most prevalent cancer and studies further show, surprisingly that breast cancer is dependent on population ethnicity (DeSantis et al. 2016). Women inhabited in Asian pacific regions possess less chance of breast adenomas compared to other regions of the globe. Life styles are shown to have a major part associated with breast cancer incidence, showing an increase of 3.2% in 2013 compared to breast cancer rates in 1970's (DeSantis et al. 2016).

(American cancer society report (2002) outline that women conceiving at their early age, lactate for prolonged periods are found resistible towards breast cancers. Awareness of self - examination of breast, mammographic detection at the early age of around 40 are highly recommended for increasing the life span and providing effective treatment in women diagnosed with breast tumour (Desantis et al. 2011). According to the recent statistical analysis by the American cancer society, the female population express a 20% less prevalence towards almost all of the cancer types, except that for thyroid tumour, which accounts for higher rate of female 3 : 1 with the male population (Siegel et al. 2017).

Population - based studies shows that breast cancers are highly associated with the age of the individual and ancestors – i.e. a strong genetic component (Ziv et al. 2006; Kamińska et al. 2015). According to the histopathological examination findings of their research, women diagnosed with benign conditions were more likely to perceive proliferative breast cancers. Their findings also infers that the chance of acquiring the condition increased with age (Hartmann et al. 2005).

The incidence of colorectal cancers was revealed to be substantially more or less the same in terms of gender, the study also relates age as an important factor which determines the tumour developing destination within the colorectal regions (Siegel et al. 2014). According to the study of prevalence of cancer in the United Kingdom alone, the incidence of cancer shows a dramatic inflation of around 2 million in the year 2010 which inclined with 400,000 more cases within a 5 year span and these figures are anticipated to rise at an alarming within the coming years (Maddams et al. 2012). As per the cancer incidence reports disclosed by the american cancer society in 2018, the incidence of the colon cancer rates were only 3% less, while leukaemia were found to be 16% lesser in females compared to the male population in the united states (L. et al. 2018).

1.2 Factors associated with cancer initiation and progression

Ancestral gene mutations passed on to successive generations are the root cause of hereditary cancer according to population - based mapping analysis. Hence these expression studies provide an explicit outline of genetic alterations which can help, not only improve the quality of current treatment strategies, but also enhances early diagnosis (Bock Schwartz et al. 2009; Chen et al. 2001).

Apart from mutations, exposure to chemical carcinogens, ionizing radiation and oxidative stress are some important factors which hampers the normal functioning of genes associated with cell regulation, eventually leading to uncontrolled cell replication**.** Besides mutation and hereditary factors, cancer stem cells as one of the main causes of reappearance of several solid malignancies. The cancer stem cells are also the root cause of emergence of bone marrow mutation resulting in leukaemia cancers (Huang et al. 2010). The colorectal carcinoma is reported to have close association with the gut microflora that is involved in the stabilization of the intestinal pH. Less levels of intestinal microflora attributed towards enhancing the pH, which is one of the factors favoring colorectal cancer initiation (Ohigashi et al. 2013).

1.2.1 Cancer and apoptosis

The term apoptosis refers to programed cell death. In healthy tissues, normally, the cells receive signals to undergo apoptosis upon reaching cellular maturation or due to unhealthy

states, to sustain a stable cell population (Wong et al. 2011). Cells are also triggered to undergo apoptosis by intracellular and extracellular responses including pathogenic intrusion, hypoxia and cellular stress responses (Susan et al. 2007). The normal cells losing its property to undergo apoptosis becomes cancerous. Avoidance of apoptosis is a major tumour property. Malfunctional status of apoptotic genes mainly tumour suppressor genes and the active involvement of anti - apoptotic signaling pathways helps in sustaining cancer cell survival. Besides this, the cancer cells express elevated levels of stress protein HSPA1A, which not only defend the cancer cells from apoptosis but also plays an eminent role in perverting cancer treatment resulted nucleic acid repairs (Kaul et al. 2011). Hence therapeutic efficiencies could be improved through inhibition of HSPA1A protein. This thesis involves studies on how cancer treatments could be made more effective by modulating apoptosis, principally by inhibition of HSPA1A and overexpressing tumour suppressor protein p21.

1.2.2 Genes associated in proceeding apoptosis

Genes in the BCL - 2 apoptotic route are strongly dependent on each other's functional status in initiating apoptosis. Among these genes include mainly PUMA, which is eminent protein in hampering the activity of anti - apoptotic molecules, thereby assisting apoptosis (Fricker et al. 2010). Studies relate the instability of this polypeptide resulted from phosphorylation at its serine 10 amino acid sequence, thus hampering the apoptotic process in cancer cells (Fricker et al. 2010). NOXA is another protein whose impaired functional status adversely affects the p53 and p21 gene directed cell death routes (Shibue et al. 2003).

In vivo study shows the active participation of NOXA not only in activating the host defense system, but also in enhancing cancer cell sensitivity towards conventional anti - cancer agents (Ploner et al. 2008). Other proteins in this apoptotic signaling network includes BID (BH3 interacting domain) which are studied to works as vehicle in driving the pro apoptotic protein Bax to the mitochondrial destination, upon reaching the mitochondrial outer core, the Bax protein accompanies the Bak polypeptide to facilitate the liberation of cytochrome c, which is an intermediate process in initiating programmed cell death through the p53 directed apoptotic pathway (Westphal et al. 2011). The up regulatory status of the Bax protein is evidence to promote apoptosis in response to phytic acid in colorectal cancer cell lines HT-29 (Shafie et al. 2013). The Bax protein is studied to be transported to the mitochondria, which

in turn disrupts the mitochondrial membrane resulting in apoptotic response towards resveratrol treatment in leukaemia cell lines U937 (Guha et al. 2011).

The role of BAD protein of pro apoptotic gene family serves the apoptotic pathway through TNF - α mediated cell death route. Studies evidence the role of these proteins being attenuated by Ikb kinase complexes in cancer cases, thus defending them from undergoing apoptosis (Yan et al. 2013; Westphal et al. 2011).

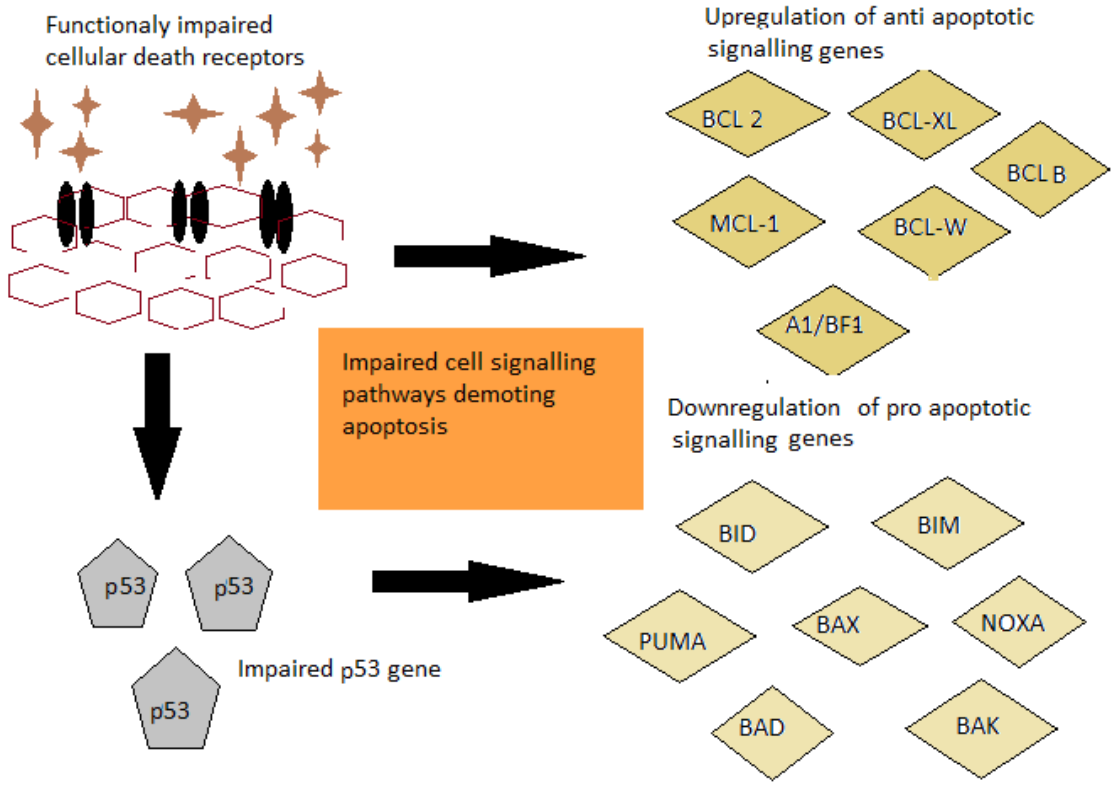


Fig 1.2 The diagram illustrates the p53 directed impaired apoptotic pathway in cancer cells. It involves the downregulation of apoptotic signaling machineries together with over expressive status of anti - apoptotic genes and above all mutation of p53 genome, thus promoting cancer cell progression, adapted from (Haupt et al. 2003) nature.com.

1.2.3 Genes associated in hampering apoptosis

The cellular programmed death signaling routes are halted by the interaction of several oncogenes thus allowing cells to become immortal leading to cancers (Figure 1.2). BCL - 2 and BCL - XL polypeptides in the apoptotic signaling network are studied to block the function of apoptotic promoting genes BAX and BAD, thus refraining the liberation of cytochrome c from the cells ATP factory, finally preventing apoptosis (Huang et al. 1998). Other genes accompanying the anti - apoptotic process in the BCL - 2 signaling cascade include MCL - 1, BCL 0 - B and A1/BF1 (Huang et al. 1998). The BCL-2 protein plays a central role in preventing apoptosis. BCL-2 knock down studies in leukaemia cell lines U937 transfected with phytochemical anthocyanins, exhibited apoptosis through the activation of the caspase cascade 3, 8 and 9, which eventually resulted in apoptosis, compared to BCL-2 overexpressing cell lines (Lee et al. 2009). *In vitro* studies reveal the suppressive role of anti-apoptotic protein BCL-XL in inducing apoptosis in response to treatment with phytic acid in colorectal cancer cell lines HT-29 (Shafie et al. 2013).

1.2.4 Heat shock genes and apoptosis

Cancer cells express escalated levels of HSPA1A proteins which plays a protective role in cancerous cells, preventing cell death. Heat shock proteins are produced in both eukaryotes and prokaryotic organisms as an evolutionary path shielding intracellular proteins from thermal denaturing (Chatterjee et al. 2017). Elevated heat exposure, ultra violet radiation exposure, and oxidative stress are some of the major factors studied to be linked with over expression of heat shock genes, mostly HSPA1A and HSP90A (Chatterjee et al. 2017).

Heat shock effec**ts** were reported to be first observed in tissues of fruit fly Drosophila melanogaster during its different pre - pupal developmental stages, their experimental inference evaluated significant variation in chromosomal puffs in tissues subjected to heat shock treatment (Ritossa et al. 1996). In mammals, Heat shock genes are categorized as 4 annexes based on their high molecular mass into, HSPD1, HSPA1A, HSP90A and HSP as low as 10 - 15Kda which comprises of HSPB1. These molecular chaperons are found to be engaged in maintain the conformational status of proteins, signaling, functioning and regulation of other proteins within mammalian cells (Jolly et al. 2000). The function of these unique peptides alters with their molecular weight and their cellular localization which are studied to

differ with cancer types (Ciocca et al. 2005). Considering their functional role within various intracellular compartments, they are classified into eight annexes, two of which include - the HSPA5 working in relation with proteins of the endoplasmic reticulum and HSP90A which is studied to be associated within the mitochondria of eukaryotic cells (Ciocca et al. 2005).

On the other hand, low expression levels of HSP60 and HSP90 were studied in high tumour status, which resulted in reappearance of superficial bladder malignancies in patients after primary surgery, thus proving the active participation of these molecules in enhancing the host immune response (Lebret. et al. 2003). And hence are studied to be good prognostic tool for patients resistant to normal cancer treatments and the risk of reoccurrences could be tackled at the early stages of the condition. These proteins are studied to be remarkable prognostic markers for not only cancers but for conditions like immune thrombocytopenic purpura in pediatric cases where antibodies against HSPA8 are detected predominantly. Hence screening of HSPPA8 antibodies makes treatment procedures far promising (Xiao et al. 2004).

Intriguingly, heat shock proteins function as alarm system, eliciting the natural defense mechanism, or in some instances, their expression works to demote natural immunity, allowing the progression of malignant cells. Cellular contents released as result of necrosis, allows the intracellular heat shock proteins reach exterior, helping trigger the immune system identify the threat and act accordingly (Klink et al. 2012), hence heat shock proteins are studied to be important proteins in activation of immune response and render tumour invasion. Several pharmaceutical trials output the positive potential of using high molecular weight heat shock proteins such as HSP110 as powerful tools in generation of cancer targeting vaccines. Hence an explicit overview of function of these proteins at intracellular and extracellular levels may help improve conventional therapeutic index in cancer patients (Multhoff. 2006; Daming Zou. 2016).

The overexpression of HSPA1A are studied to be key factors associated to cellular proliferation in most of the malignancies, although scientists have introduced several drugs which hamper the function of these genes, recent findings reveals that, the transcription of HSPA1A can be managed indirectly by attenuating the intracellular link mediated through several other factors such as NEF - Nucleotide exchange factors and TPR - Tetratricopeptide

chains, which works in close connection with HSPA1A protein resulting in insensitiveness to natural host defense, promoting cancer survival (Assimon et al. 2013). The HSPA1A within an extensive network indirectly hinders the transcription of apoptotic factors such as BAX, and cellular death accelerating systems DR4 and DR5 (Figure 1.3), promoting cancer proliferation (Guo et al. 2005; Murakami et al. 2015).

Intriguingly, cancer cells are guarded by HSPA1A proteins from the host cell natural defense mechanism exerted by TNF - α (Tumour necrosis factor), thereby preventing cellular necrosis (Daugaard et al. 2007). HSPA1A deregulates the cells apoptotic process by engaging themselves in reverting the segregated proteins conformational status and switching back to their function mode (Murphy. 2013).

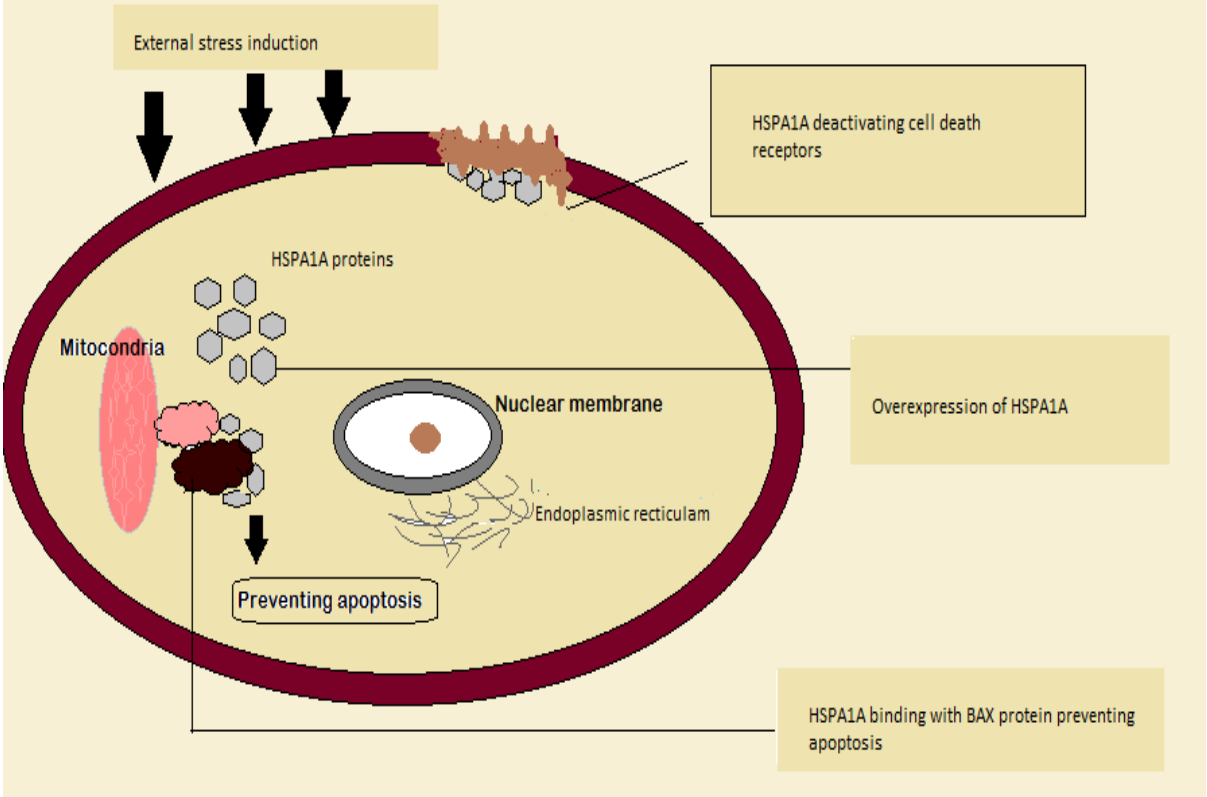


Fig 1.3 The diagram illustrates the involvement of stress induced HSPA1A protein in preventing apoptosis. The overexpressed proteins bind to death cell receptors and apoptotic protein BAX, hampering their role in initiating the apoptotic process, adapted from (Wang et al, 2012) and from the data demonstrated in this thesis.

1.2.5 Interactive role of tumour suppressor gene p53 and p21 in cancer

Researchers point out that half of the global cancers are resulted due to the impact of alterations of p53 genome in cancers. One of the important factors known as histone acetyltransferase (HAT) enzymes are studied to be involved in the acetylation of lysine amino acid residues to form N - acetyl lysine, which is a necessary for the transcriptional activation of p53 gene (Wright et al. 2016). Despite being a tumour suppressor in function, p53 under altered status, replace a negative role as a tumour accelerator, which is notified to be main cause of cancer insensitivity towards several therapeutics (Goldstein et al. 2011; Parrales. 2015).

Report suggests that p53 expression levels are counterbalanced in cells by certain proteins referred to as ESTIC, which encounter a major role in assisting p53 gene mediated repair of nucleic acids (Luo et al. 2016). *In vitro* ESTIC silencing study reflected the malfunctional status of p53 gene is due the absence of these unique factors which play a key role in degradation of mutated p53 mRNA, thereby preventing cancer cells resulted from DNA damage from undergoing apoptosis (Luo et al. 2016). Hence, most of the cancer cases can be addressed by implementing therapeutic strategies focussed at ablation of non - functional p53. Although, tumour suppressor gene p53 is designated by the scientific community as the guardian of the DNA double helix, the cyclin dependent kinase inhibitor p21 functions in connection with p53 tumour suppressing network arresting the cancer cell growth and metastasis. More explicitly stating, in the p21 mediated apoptotic pathway, p53 fulfils its tumour inactivation by binding to p21 gene which in turn binds to CDK1, CDK2, CDK4 and CDK6 complexes, arresting the cell cycle at the G1 phase (Fischer et al. 2015). Natural cell deterioration widely known as senescence resulting from external or internal cellular stress is an important factor involved in replication arrest. This mode of cellular growth arrest is assisted through tumour suppressor p53 (Schmitt et al. 2002).

Phosphorylation of p53 at its serine residue evidenced to elevate p53 expression in response to anti-cancer agent curcumin, which in studies highlight the active role of JNK signalling pathway in promoting p53 independent cell death in response to chemotherapeutic cisplatin in U937 leukaemia cancers (Bae et al. 2006). Hence the anti-cancer mechanism exhibited by

JNK may be useful therapeutic target for cancers devoid of p53 functional status. Moreover, the phosphorylation status of p53 is also crucial in p53 mediated apoptosis. Phosphorylation in turn transactivated anti-apoptotic factors Bax, resulting in programmed cell death in colorectal cancer cell lines HT-29 (Song et al. 2005).

Interestingly elevated expression of p21 genes are studied to accelerate programmed cell death in response to chemotherapeutic treatment, thus displaying the eminent role of p21 gene in a wide range of malignancies. Adenoviral mediated gene transfection scientific reports support the potency of p21 gene in arresting colorectal progression and metastasis (Wang et al. 2015).

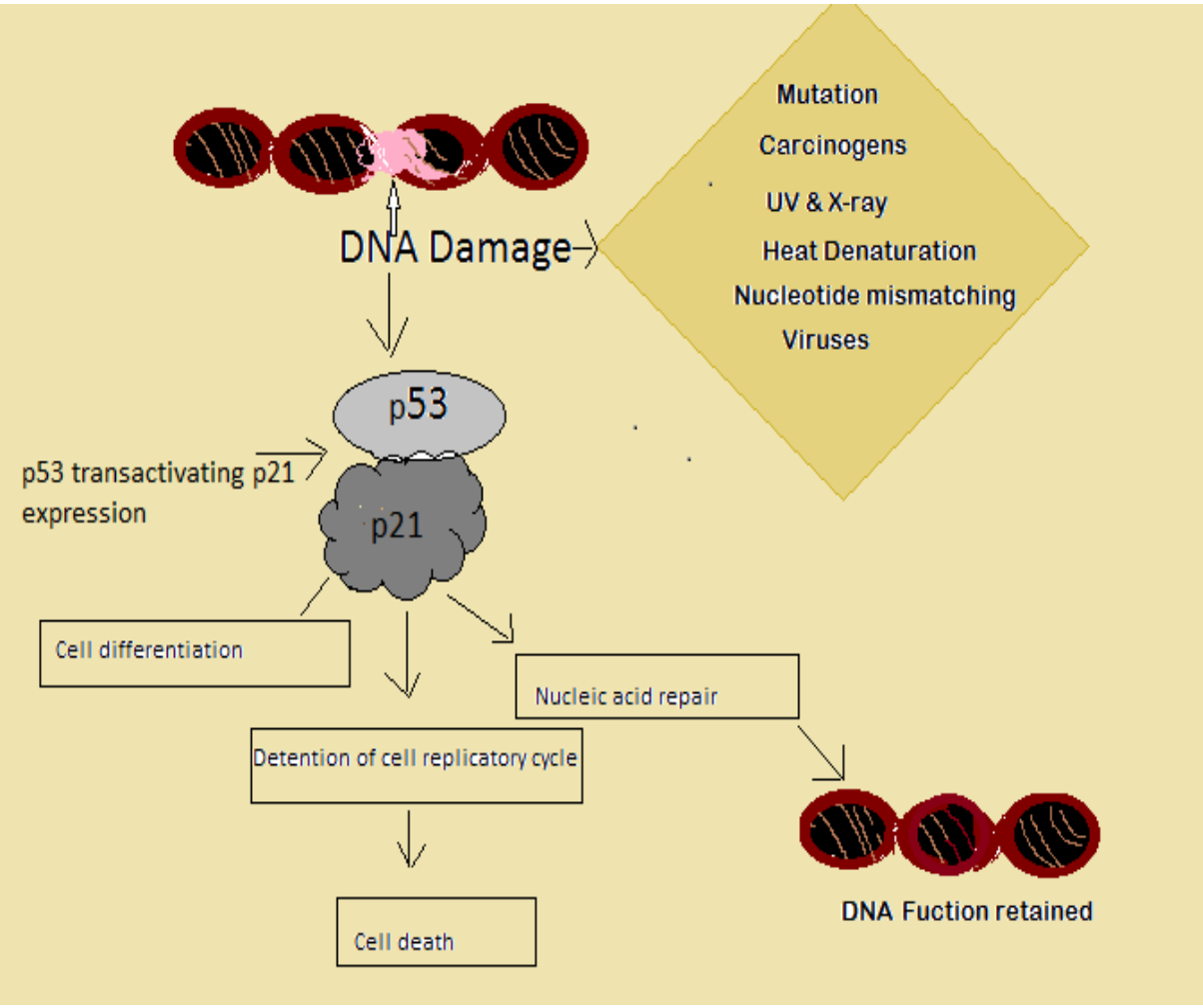


Fig 1.4 The diagram depicts the tumour suppressor gene mediated DNA repair. The figure displays how DNA damage due to radiation, mutation, infection or carcinogens are repaired by the active involvement of the tumour suppressor genes p21 and p53 signaling cascade in initiating apoptosis, adapted from (Fischer et al. 2015; Shibue et al. 2003).

1.2.6 Genes associated with p21 upregulation

The status of p21 activity relies on its position in cellular compartments, which is the main factor which determines its function as tumour promoter or tumour suppressor. Besides genes that promote cancer cell progression and metastasis, there are several signalling proteins which act as switch boards in cancer cell control and regulation (Figure 1.4). p21 gene is studied to be accelerated in colorectal cancers by various pathways, one such route is MEK/ERK - signalling network. Although MEK/ERK pathway directs extracellular signals to the DNA for transcription and replication, allowing cancer progression, this pathway is studied to induce nuclear p21 expression *in vitro* in colorectal cancer cell lines HT-29 subjected to treatment with zinc (Park et al. 2002). Moreover, the downregulatory status of anti-apoptotic proteins BCL-2 and survivin are closely linked with enhancing the expression of tumour suppressor protein p21, which evidenced to induce apoptosis in leukaemia cell lines U937 transfected with 12 - O - tetradecanoyl phorbol -13 acetate (TPA (Luo et al. 2005). The transforming growth factor TGF - β also plays a key role in increasing the expression of p21, which in turn bind the CDK complexes, arresting the cell cycle at G1 phase. TGF - β works through SMAD signalling cascade boosting the level of p21 initiating cell cycle arrest (Bauer et al. 2015; Padua. 2009).

Reports point out that p21 genes are counter controlled by microRNAs in achieving their apoptotic role in colorectal cancers. *In vitro* transfection of MIR 106 and MIR 13a evaluated their active requirement in the p21 apoptotic cycle in the presence of butyrate (Schlörmann et al. 2015). Moreover, *in vivo* MicroRNA transfection experiments evaluated the inhibition of MIR 708 - 5p which resulted in elevated p21 expression levels, suggesting the association of MIR 708 - 5P with the cyclin dependent kinase inhibitors signalling cascade initiating tumour suppressive properties in adenomas of lungs (Wu et al. 2016).

Hence, cancer treatments can be modified by implementing drugs that target microRNA - 208 and MIR708 - 5P, enhancing survival rates. The receptor tyrosine kinase network communication in cancer cells are studied to be coordinated by genes collectively designated as SPRY, referred to as sprouty (Zhang et al. 2016). The cyclin dependent kinase inhibitor gene switchboard is interlinked to the expression of SPRY genes. *In vivo* and *in vitro* studies support the demotion of colorectal carcinoma upon declined expressive status of sprouty genes (Zhang et al. 2016).

1.2.7 Up regulatory status of p21 in chemotherapeutic induced cancer arrest

Reports have shown increased expression status of p21 protein in several cancers in response to apoptosis induced by conventional chemotherapeutics including genistein, lysophosphatidic acid etc (Majid et al. 2008; Wu et al. 2011). Apart from FDA approved chemotherapeutics, commercially available antibiotics including ascocholrin and ascofuranone have also proved to possess not only antibacterial properties, but also are promising anticancer agents according to latest *in vitro* report (Jeong. 2010). *In vitro* study emphasize the promising therapeutic index of several iron chelating agent such as Dp44mt (Rao et al. 2009). Chelating effect varied with cancer type. p21 expression was observed to be elevated in pancreatic and melanoma malignant tissues exposed to Dp44mt. On the other hand, Dp44mt evidenced a declined p21 expression in breast cancers (Rao et al. 2009).

Hence these anti-cancer agents are found to be promising treatment strategies towards p53 mutant cancer treatments and safe to use as they are proved non - toxic through *in vivo* investigations (Rao et al. 2011; Rao et al. 2009). Besides, chemotherapeutics, dietary fibre content helps as a defensive factor in colon tumour, as they release an important compound called butyrate which abstain anti - tumour progressive properties. Translation of p21 protein is studied to be an inevitable factor in assisting butyrate provoked cell death in colorectal cell lines HT-29 (Schlörmann et al. 2015; Archer et al. 1998). p21 is also reported to be an upstream regulator of apoptosis mediated through caspase - 3, 8 and 9 in leukaemia cell lines U937 transfected with grape seed extracts (Gao et al. 2009).

1.3 Role of p21 in cell cycle regulation and HSPA1A

The tumour suppressor gene p21 plays a major role in controlling the eukaryotic cell cycle in response of several factors including DNA repair, cellular inflammation and oxidative stress. p21 mediates this regulatory function by directly inhibiting the function of cyclin dependent kinase complexes Cdk 2,4 and 6 at the G1 phase, Cdk2 at the G2 phase and PCNA - cell proliferating antigen at the S phase, thus preventing their role in DNA replication, finally arresting cell cycle progression (Gartel et al. 2002). The function of p21 exclusively dependent on the phosphorylation of p21 protein at different sites, which in turn determines its function in halting the cell cycle check points. Reports also show that phosphorylation of p21 at threonine 145 prevents p21 assisted PCNA inhibition, preventing DNA synthesis (Li et al. 2002).

Besides the phosphorylation status of p21, its localization within the cell compartments determine its function as positive or negative regulator of cell cycle. The presence of this protein in the nucleus presents its function in negative regulation of cell cycle, while its location in the cytoplasmic region reflected its role in tumour promotion (Xia et al. 2004). Moreover, studies reflected the degradation of p21 by E3 ubiquitin ligases, which in turn compromises the stability of p21 with in cells.

Intriguingly, the anti-proliferative stress response mediated cellular arrest are studied to be overridden by the counter protective role of heat shock protein HSPA1A in eukaryotic cells (Daugaard et al. 2005). The heat shock protein HSPA1A function in binding to and re-establishing the functional status of impaired proteins, thus prevented cell cycle arrest. Reports have stated the inhibitory status of HSPA1A not only in enhancing cellular arrest but also suggesting its inhibitory status in sustaining cellular stability (Daugaard et al. 2005). Studies have evidenced the active binding of HSPA1A with the tumour suppressor protein p53, which prevented the interaction of HSPA1A from repairing damaged proteins, resulting in enhanced cell death in cancer cells (Elengoe et al. 2015). However, no studies have elucidated the interaction of HSPA1A and p21 proteins.

1.4 Apoptosis and necrosis in eukaryotic cells

In eukaryotic cells, the process of apoptosis, which is considered as programmed cell death, through which the cells undergo dramatic changes morphologically, form apoptotic vesicles comprising aggregates of the mitochondria, ribosomes and condensed nucleus bound within the rigid cell membrane as illustrated in (Fig 1.5 A). Whereas in case of necrotic cell death, the cell membrane ruptures releasing the disintegrated cell organelles as illustrated in (Fig 1.5 B). Finally, the cells that undergo death by either of the pathways are eliminated from the system by the process of phagocytosis (Fink et al. 2005).

The stress protein HSPA1A has been reported to have strong interaction with the apoptotic pathway, hampering cell death. The apoptotic pathway in brief involves the participation of several signaling molecules that include mitochondrial product - cytochrome c which upon binding with Apaf - 1, finally generates apoptosome, which is the fundamental element to initiate the apoptotic process. HSPA1A play an anti - apoptotic role in hampering the

generation of apoptosome, thus protecting cancer cells from undergoing apoptosis (Beere et al. 2000).

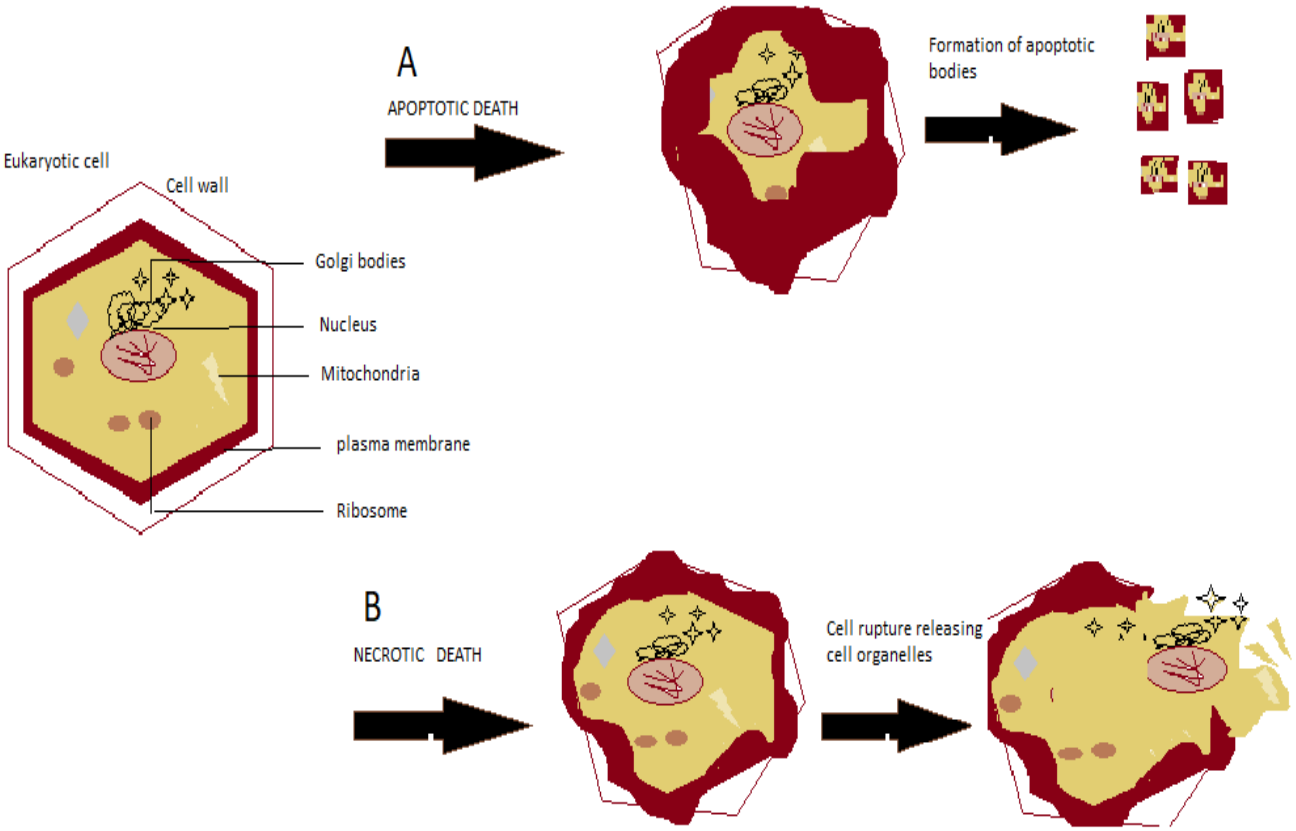


Fig 1.5 The diagram illustrates eukaryotic cell death routes (A) Apoptotic cell death which involves the formation of apoptotic vesicles comprising of cell organelles enclosed within rigid cell membrane; (B) The necrotic death route in which the disintegrated cell organelles are released to the cell exterior; adapted from elsaivadoria.com; (Fink et al. 2005).

1.5 Cancer treatments and issues addressed

1.5.1 Surgery

Cancer at it's initial stage is highly localized small tissue outgrowth. This tissue mass increase in size reaching its secondary cancer stage. The cancer cells eventually migrate to surrounding tissues and reaches the stage of angiogenesis – the third stage of cancer. Once the cancer enters the circulatory stream, it spreads uncontrollably. Tissue resectioning is a successful cancer treatment for benign tumour and solid malignancies until it breaches the stage of angiogenesis. Once the cancer is metastasized to the surrounding tissue regions, surgery

along with adjuvant chemotherapy or radiation therapy is administered, refraining the cancer from further proliferation. Surgery is recommended as first phase treatment of colorectal cancers, followed by adjuvant therapies administered to prevent future reoccurrence (Dorudi et al. 2002). Surgery on the otherhand is not suitable treatment strategy for leukaemia cancers as the cancer cells are localized in the circulatory blood stream.

1.5.2 Radiation therapy

Treatment of cancerous tissues with ionising rays such as X - rays, ultra violet rays independently and in combination with chemotherapeutics accounts for more than half of the cancer survivals. Undesirable side effects generated due to radiation exposure is of high consideration, hence this issue needs to be tackled by modulating this treatment approach more tumour precise, expressing less side effects. The radiation waves results in direct accumulation of energy in the exposed tissues, creating multiple breaks in the nucleic acid resulting in cellular death irrespective of cancerous or healthy tissues (Maier et al. 2016). Despite inducing DNA destruction, the effects of radiation rays are studied to activate the transcription of several anti - apoptotic genes. These genes include mainly the AKT/P13 signalling pathway, which are closely associated with the transactivation of enzyme DNA dependendent protein kinase, which assist non - homologous repair of broken stands resulted from radiation therapy (Schuurbiers et al. 2009). Hence, this anti - cancer signalling pathway play a crucial role not only in defending cancer cells from undergoing programmed cell death, but also studied to be the main cause for emerging insensitiveness to radiation therapy (Hein et al. 2014). Other genes including the members of mitogen activated kinases (MAPK) are also evidenced to protect cancer cells from death responses from radiotherapy (Dent et al. 2003). The underlying molecular mechanisms behind the enhanced transcriptional status of MAPK genes upon radiation exposure varied with the cancer type, with various factors in close connection including the cellular growth signalling members of the P13 kinases and autocrine mechanisms (Dent et al. 2003). Hence, there arises the necessity to develop treatment modalities capable of attenuating these anti - apoptotic signalling networks, which may enhance the therapeutic index of radiation - based cancer treatments.

1.5.3 Chemotherapy

Chemotherapeutics are potent in triggering apoptosis in cancercerous tissues due to their

active participation in enhancing expression of tumour suppressor genes, inhibition of heat shock proteins, BCL2, ATG5, coupled with low expression status of VEGF genes (Lai et al. 2012). Cancer therapeutics such as ginsenoside are reported to actively release the immune T - cell effector molecule IFN – γ, which resulted in immune response induced apoptosis in melanoma and colon cancers (Keum. 2016). Moreover, Ginsenoside treatment also showed elevated expression of heat shock proteins HSP60, HSP70 and HSP90 which reflects the active participation of these proteins in apoptosis induced by ginsenoside (Son et al. 2016). However, one of the major concerns in using chemotherapeutics is that it destructs healthy tissues as well.

1.5.4 Combination therapies

Due to gradual resistance addressed by 5 - fluorouracil administration, combined treatment of 5 - fluorouracil with other chemo agents like leucovorin, methotrexate, oxaliplatin and irinotecan has paved the way for improved treatment outcomes in advanced cancer stages. However, in relapsed colorectal cancer conditions, 5 - fluorouracil fails to produce the expected treatment outcome, presenting 80-85% of drug resistance (Boige et al. 2010). This insensitiveness of cancers towards conventionally available chemotherapeutics confines to poor survival rates, which is the major problem encountering cancer treatment.

Research studies show that combining 5 - fluorouracil with curcumin, which is generated from the root portion of turmeric plant accelerates anti - metastatic properties, initiating cytotoxicity in HCT - 116 colorectal cancer cells which were found insensitive to 5 - fluorouracil treatments in its intact form (Shakibaei et al. 2015). Cancers irresponsive to intact 5 - fluorouracil depicted significant cytotoxicity upon treatment with curcumin at limited dose as low as 5μM of 5 - fluorouracil in original HCT - 116 cell lines (Shakibaei et al. 2015). Curcumin in combination with anti-cancer agents have shown accelerated effects compared to chemotherapeutics in its intact form (Shakibaei et al. 2015; Giaccone. 2004). Curcumin in combination with 5 - fluorouracil works through strict management of thymidylate synthase, which plays a major role in DNA repair and replication process, in turn blocking the function of nuclear factor kappa in breast cancers (Tian et al. 2012; Vinod et al. 2013). Drug combination trial including irinotecan and 5 - fluorouracil have increased the treatment efficacy in highly relapsed cancer cases, most effectively in colorectal cancers (Rothenberg.

2001). Clinical trials focused on implementing irinotecan together with cisplatin administration reflected improved therapeutic index in various malignancies involving small cell lung cancers (SCLC) and non - small cell lung cancers (NSCLC). Although several studies reveal the activity of topoisomerase 1, the mechanism of irinotecan remains unclear. An insight into exact cellular activity by irinotecan administration, and the causes of drug insensitivity may throw light in designing cancer therapeutic like irinotecan in a more target specific manner, making it a complete chemotherapeutic (Rothenberg. 2001).

Although conventional treatment strategies arrest cancer growth, these treatment approaches are found to express side effects and so are deleterious to the healthy cells. Moreover, in relapsed cancer conditions, these treatments tend to become less responsive, leading to increased mortality rates. Hence in this present scenario, there arises the necessity to establish highly responsive treatments with confined side issues.

1.5.5 Hyperthermia treatment

Besides, chemotherapy and radiation therapy, heat therapy is also an effective treatment modality for cancer. Hyperthermia at temperature range within 45℃ evidence cancer cell destruction, even in deep tissue destinations (Hildebrandt et al. 2002). Cancer cells disrupted due to heat induction, release damaged proteins to the cell surface, which act as alarm signals, by which the host immune cells detect the threat and act accordingly (Skitzki et al. 2009).

Interestingly, administration of hyperthermia has reported to enhance the therapeutic potential of cancers. Hyperthermia evidence to work through ubiquitination of the FLIP protein which is involved in modulating death receptor signalling, directing the transactivation of the mitochondrial apoptotic pathway resulting in apoptosis in colorectal cancers (Song et al. 2013). Studies also reflected the key role of hyperthermia in combination with as β - elemene which mechanistically arrested the cell cycle at the S phase, enhancing the transcription of apoptotic factors p21 and BAX genes in accelerating apoptosis (Wu et al. 2017). Their findings also reflect the active participation of hyperthermia in suppressing further proliferation of cancer cells, enhancing the therapeutic index of lung malignancies (Wu et al. 2017).

Moreover, adjuvant administration of hyperthermia with currently practised treatments play a major role in rendering the natural self - rectifying mechanism, preventing pro - survival of cancer cells (Oei et al. 2015). Thus, allowing treatment modalities to overcome resistance towards conventional cancer therapeutics (Oei et al. 2015). Report show the active participation of hyperthermia in inducing apoptosis through caspase - 3 activation and increasing the expression of p38 in adjuvant therapy using baicalin in leukaemia cell lines U937 (Zakki et al. 2018). Moreover, hyperthermia effect could also be boosted by the effect of antioxidant such as melatonin, which can activate the mitochondrial apoptotic signalling factors in Leukaemia cell lines U937 (Quintana et al. 2016).

The side effects generated due to conventional cancer treatment strategies often weakens the host system. Studies also point that hyperthermia in combination with conventional treatments help in eliciting host immune responses, which is eminent in defending cancer cells from further proliferation post conventional treatment strategies. Hence the destructive impact induced by hyperthermia helps overcomes the resistance experienced with conventional treatment and the confined side effects of hyperthermia to non - cancerous tissues makes it an excellent treatment strategy for cancer treatment (Ahmed et al. 2013).

1.5.6 Natural anti-cancer agents

Apart from conventional cancer treatment strategies, naturally occurring phytochemicals are also experimentally proved to have effective anti-cancer properties. Over the years, studies have showcased the cancer therapeutic efficiency of individual application of herbal plant parts individually. However recent reports highlight the therapeutic potency of using the whole plant essence of Asteraceae and Urtcaceae (Solowey et al. 2014). This report highlights the effectiveness of Urtcaceae herbal contents in not only inducing apoptosis, but also in rendering further proliferation of breast malignancies. Moreover, these herbal essences are proved to be highly cancer targeted, hindering the healthy tissues from its cytotoxic effects (Solowey et al. 2014).

Besides herbal anti-cancer agents, chemical compounds enriched in foods including broccoli and watercress are evidenced to have profound anti-cancer properties capable of arresting the roots of colorectal carcinomas (Pereira et al. 2017). *In vitro* studies reveal the therapeutic potential of phytochemicals enriched in curcumin, which play a major role in blocking the

construction of new circulatory routes within cancer cells, finally suppressing metastasis (Shao et al. 2002). Apart from curcumin, vitamin D also shown to disrupt the function of receptor tyrosinase activity, which in turn repress cancer growth, angiogenesis and induce programmed cell death in breast, prostate and colorectal malignancies (Campbell et al. 1997; Chakraborti et al. 2011).

1.6 Strategies in improving the therapeutic potential of cancer therapies

1.6.1 Intracellular delivery entities - nanoparticles and drug delivery

Although conventional therapies including chemotherapy are effective treatment strategies against cancers, the side effects generated due to high dose administration, poor drug bioavailability and chemo - resistance are some of the issues encountered by these treatments. Hence, these issues could be resolved with the use of nano-based drug delivery systems, which are becoming powerful and promising platform for cancer therapies. Nano elements modulated with specific surface ligands such as polyethylene glycol with nano scale ranging from (1nm to 100nm) provides direct access towards precise tumour destinations within biological systems enhancing effective drug delivery (Davis et al. 2008). Moreover, studies show that nanoparticles known as nanoflares introduced into the circulatory stream helps bind the cancerous cells due to their selective cancer target binding property resulting in light emission upon reaching the cancer destination. Hence, these agents are excellent diagnostic tools, thus promoting opportunities for developing new perspective cancer therapies (Davis et al. 2008).

Nanoparticles engineered from different sources such as metals, liposomes, dendrimers, nucleic acid, protein and polymer conjugate nano particles are explored to have therapeutic application as drug carrier platforms in medical oncology (Zhang et al. 2008). As far as drug delivery is concerned, the most important aspect is the selection of nanoparticle, taking into concern, its toxic free nature, high rate of biodegradability, prolonged circulation time and targeted drug delivery application within biological systems. These properties of nanoparticles help overcome certain limitations of conventional therapy such as reduced drug bioavailability, high dosage induced toxicity and developing drug resistance together with lack of specificity affecting both cancerous and healthy tissues irrespectively are few of the issues encountering traditional cancer therapies. Studies interprets that iron nanoparticles from

degraded spions, which comprise superparamagnetic properties are absorbed naturally by hemoglobin, the iron reservoir of the human system (Nakamura et al. 2013). Moreover, the super magnetism provides magnetic resonance imaging, which helps to quantify the iron accumulated in the brain due to disease conditions such as multiple sclerosis, helping clinicians diagnose cancer status and to modify treatment procedures accordingly (Nakamura et al. 2013). The therapeutic and diagnostic application addresses spions as promising candidates for effective delivery and tracking of biological anti-cancer agents to intended tumour regions and brings hopes of developing highly potential and safe personalized cancer therapies, reducing cancer mortality.

Magnetic nanoparticles are competent systems possessing diagnostic as well as therapeutic value. The magnetic nano spheres function to elevate the normal body temperature within cancer tissues, a mechanism known as hyperthermia, resulting in DNA damage, making tumour tissues more susceptible towards conventional chemotherapy and radiotherapy (Giustini et al. 2010). These magnetic particles possess electro - magnetic energy, once entering a magnetic undergo oscillatory movement releasing heat high enough to degrade proteins associated with tumour progression (Giustini et al. 2010).

The sizes of magnetic nanoparticles are of great consideration in creating the desired hyperthermia effect. The smaller the particle size the more effective will be the hypothermic effect and lesser will be the interaction between particles, which in turn prevent particle aggregation. On the other hand, as the particle size increases, the particles fail to cross biological barriers within cancer tissues, ending up in particle aggregation, which are studied to cause deleterious effects such as hemostasis (Bañobre. et al. 2013).

Taking together the issues generated due to size distribution of these magnetic nanoparticles, researches are now focusing at modifying the outer surface of these nano spheres with biological polymers or site-specific antigenic receptors, which enables specific targeting of cancer cells and initiate hypothermic effect (Manual Baobre. 2013).

Metals nanoparticles of gold, silver, iron and copper are excellent nano carriers in the field of biotechnology. Scientists are evaluating the intracellular dispensation of gold nanoparticles within human systems using computer system - based research. These computational analyses may provide access to information regarding not only bio - distribution of nano

elements, but also helps in assessing the toxic nature of nano elements, which is to be taken into high consideration in developing safe drugs (Cheng et al. 2018). Apart from various chemo - agents, doxorubicin produces expected results in primary lung cancers, doxorubicin together with other chemo - agents are also applicable for relapsed cancers. Doxorubicin packed within nano - delivery systems of poly (butyl cyanoacrylate) was developed by researchers to overcome the poor drug bioavailability within circulatory system to produce desired cytotoxic activity within lungs (Consolacion et al. 2015).

Statistical analysis reveals pancreatic cancers as a major disease presenting increased mortality rates (Del Chiaro et al. 2014). Gemcitabine is widely accepted drug administered for pancreatic cases. Although the drug shows desired cytotoxicity against pancreatic cancers, the problem encountering gemcitabine drug involves poor bioavailability due to very confined drug retention rate of up to 8 minutes within circulation (Kevin Affram et al. 2015). Researchers are focusing at improving the therapeutic potential of this drug by designing the drug, encapsulating the drug in liposomal delivery vehicles, to enhance prolonged drug circulation time to produce expected cytotoxicity towards pancreatic carcinomas (Kevin Affram et al. 2015).

Ibuprofen an anti - inflammatory drug which is administered normally through oral routes were framed with chitosan polymer together with glutaraldehyde reagent, emulsified with equal volumes of toluene, this clinical study aimed at bringing low gastric aftereffects normally developed due to prolonged Ibuprofen intake (Kcofokansi. 2013). The study presented expected results showing substantial drug output from the micro - system preventing undesirable effects caused as result of ibuprofen in its intact form (Kcofokansi. 2013).

Using felodipine as a model drug, Jung. A. Ko and group demonstrated the difference in drug output from microparticles generated by ionic cross - linking process, their data evidenced to have high impact in iso - electric nature of the system (Jung.A. KO. 2014). The pH of the cross-linking mixture increased together with drug liberation rate. Moreover, their finding also suggest that drug encapsulation and liberation is not only solely dependent on high pH and concentration of the cross - linking agent, but also based on the low molecular weight of chitosan molecule as low as 1% in concentration.

In human systems, liver is the main organ which plays the eminent role in removal of unwanted by - products, and so will be the final settling destination of retaining nano - elements in nano - drug administrations. A systemic clearance is required to avoid un-necessary accumulation of these nanoparticles in liver and other body parts, to prevent other nanoparticle - based inflammations and disorders. A nano drug administration experimental investigation evidenced a systemic release of cyano - acrylic nanosphere released by living systems naturally. The size distribution of the nanoparticles displays the major part in exerting toxicity. The smaller the dimension the higher is the toxic effect. Researchers exhibit that human lymphatic tissues are capable of gripping nanoparticles at surface area less than 100 nano - meter rapidly than intestinal tissues, revealing that toxicities are borne to be likely affected towards lymphatic cells by low nano - scales delivery systems (Jafar Ai et al. 2011).

One of the major problems hampering chemotherapeutic administration are confined with the side - effects. Second issue is about poor drug retention of these agents under circulation, considering precise drug release and bioavailability. In case of 5 - fluorouracil treatment, researchers framed this drug within liposomes for a site-specific continual release performance. Their results evidenced a gradual release from the delivery system, providing enhanced bio - availability together with enhanced cytotoxicity. This *in vitro* assessment related drug release to the pH of the environment and the drug release was higher at acidic pH and reduced release was observed at alkaline pH (Ofonime Udofot. 2015).

Together with improved drug retention, a systemic clearance of drugs from human system is equally important. Taking this aspect into concern, bio - degradable entities are interesting for drug designing. In various processes, various types of cross-linking polymers are used, that define the swelling capacity and size of the particles, encapsulation efficiency and bio-availability together with temperature and pH control of systems.

Moreover, considering the aggregation of nanoparticles after drug delivery, a clear understanding of their biological clearance is a necessity to introduce them as safe therapeutics within living systems. Hence in this present scenario, due to the increasing resistance to conventional treatment approaches, there arises the necessity to establish highly responsive tumour targeted drugs with confined side issues.

1.6.2 Carbon nanoparticles in clinical research

Cylindrically modified allotropes of carbon are interesting candidates for accommodating large volume of therapeutic molecules. Therefore, several clinical trials subject to application of these nano tubes in cancer therapy are at the merge of clinical phase trials. In cases of Paclitaxel administration in cancer treatments, since the drug has poor bioavailability and solubility, they are rendered from producing the desired effect (Mahmood et al. 2009). Carbon nanotube mediated chemotherapeutic delivery, presents caspase - 3 activation, inducing apoptosis in pancreatic and brain tumour cell lines, the results suggest structural deformation and nuclear separation upon treatment with CNT agents, which eventually lead to programmed cell death (Mahmood et al. 2009; Liu et al. 2008). Researchers have evaluated the positive potential output of gold nano elements embedded in carbon nano tubes using different linkage systems. According to their experimental analysis multivalent carbon nano-systems processing hydrazone linkage can release the delivery entity in environments entitled to have acidic pH levels (Jian Li et al. 2015).

Our scientific community has proven *in vitro* and *in vivo* studies, highlighting the positive output of CNTS mediated drug transport entities for effective drug response at very limited doses. But considering, the safety of implementing these nano - elements within biological systems, the negative impacts which are likely to be produced by these drug delivery systems towards human body and the environment has yet to be thoroughly examined and are still of high concern. Several nano - toxicological studies suggest the toxic aftereffects exerted by these nano molecules towards introducing respiratory disorders (Buzea C et al. 2007).

1.6.3 Chitosan nanoparticles - properties and applications

The exoskeleton of crustaceans possesses a component known as chitin, which is decalcified and deacetylated to extract chitosan, an efficient polymeric carrier system for the delivery of chemotherapeutic drugs (Ahsan et al. 2017). Chitosan molecules are highly degradable. Their biocompatible nature, together with scarce toxicity and high target accessing potential designate chitosan nano - combinations outstanding in drug delivering applications (Kalpana. 2010). Chitosan is predominantly soluble in organic acids and it confers a positive charge enhancing electrostatic interactions, which is necessary for nanoparticle formation within colloidal systems. The mucus adhesive characteristic feature of chitosan confines it not only

to cancer drug deliveries but also for ophthalmic conditions, in which drugs are required to be transported across the mucosal barriers (Artursson. 1994).

The molecular weight of chitosan molecule plays a prominent role during the degradation process within biological systems. The lower the molecular weight the easier will be the degradation. In cases of ophthalmic disorders, chitosan nanoelements after drug delivery undergo degradation by the enzyme lysozyme present abundantly within ocular areas (Maria Jose et al. 2003). Hence low molecular weight chitosan are selective natural polymers for drug transport applications.

Chitosan molecules are excellent carriers of proteins as the stability and bioavailability of proteins are well maintained upon chitosan encapsulation. Studies shows that proteins such as insulin embedded in chitosan polymer matrix were well insulated from gastric fluid degradation, promoting greater drug retention and controlled release for prolonged time duration (Zhang. N. 2010). MicroRNAs are studied to play an eminent part in knocking down proteins associated with prostate cancer progression. A recent study highlights that introducing chitosan encapsulated MiR-34a, depicted a massive decline in proliferative activity in bone metastatic prostate cancers in a xenograft model compared to that of existing FDA approved drugs for such cases (Sanchaika et al. 2015).

Apart from the aspect of drug delivery application, chitosan is investigated to have good potential as an antimicrobial agent. Report refer to the amino - groups in chitosan responsible for hindrance of microorganisms (Anders y et al. 2008), however, the anti -microbial activity of the compound is still inexplicit. Although 5 - fluorouracil is a broadly accepted chemotherapeutic for head, neck, pancreatic, breast cancers and highly recommended therapeutic for metastatic colorectal carcinomas, however it is bound to express less therapeutic index combined with higher issues including cardiovascular toxicities (Llorca Ferrandiz et al. 2005; Focaccetti et al. 2015).

Hence Research studies are now focused at improving the bioavailability of these fluropyramidines through induction of nano - technology. Chitosan and alginate are naturally occurring toxic free molecules which are studied to be successful drug carrier entities (Ciofani et al. 2008). The acid pH nature of the drug encapsulated chitosan matrix directs the nano sphere to release drugs at precise tumour destinations, setting free the healthy tissues from

the deleterious side effects exerted by the drug upon independent administration (Bhattarai et al. 2010).

Gefitinib is another extensively used chemotherapeutic which encounter its key participation in repressing the task of epithelial growth factor receptors, which in turn leads to programmed cell death in epithelial cancer cells. But the systemic absorption of gefitinib is considerably poor upon direct administration, therefore, studies are aiming at improving the bioavailability of gefitinib by entrapping in nano - sized carrier systems for pronounced drug effect at desired tumour destinations (Cappuzzo et al. 2005).

1.6.4 Bovine serum albumin (BSA) nanoparticles

Engineering toxic free protein nano - systems evidence to increase the therapeutic value of chemotherapeutics. BSA is one such entity, which is studied to be highly stable, with greater entrapment capacity and excellent biocompatibility and toxic free nature making them human friendly drug transporter compared to other natural polymers employed for drug deliveries (Yu et al. 2014). Moreover, BSA proteins are cleared out explicitly naturally from our human system by means of proteolysis. Hence, the application of this protein as a carrier molecule is widely accepted.

Apart from bovine serum, egg white and human serum are reservoirs of albumin. Albumin is unique in its high solubility in water, blood and organic solvents like sodium chloride. The main role of the protein is associated with balancing osmotic pressure and transport of endogenous entities within cells. Studies evidence that albumin proteins unlike other proteins withstand structural stability even if exposed to temperatures as high as 60℃. This temperature resistive nature of this molecule makes them attractive drug carrier systems (Takeda et al. 1989).

In patients suffering from inner ear illness, drugs administrated, upon travelling through the eustachian channels undergo disorientation, failing to reach the inner ear to perform desired treatment. Zhan Yu et al developed a novel nanoparticle system via heat denaturation, for treating infections associated within inner ear, using BSA for transporting Rhoda mine - B, a drug conventionally used for inner ear disorders. *In vivo* findings in guinea pigs evaluated by scanning electron microscopy and fluorescent imaging techniques exhibited prolonged

retention and penetration of the Rhoda mine - B - BSA loaded nanoparticles within the inner ear, producing expected drug effect (Zhan Yet al. 2014).

In an *in vitro* research with gefitinib loaded folate encapsulated BSA nanoparticle in brain tumour, these nanosphere unlike free drug administration, exhibited their efficient participation in knocking down LC3 - microtubule - associated protein light chain -3, together with caspase activation in cancer cells initiating apoptosis (Yijie Shi et al. 2014).

1.6.5 Lipid nanoparticles and transfection

Lipid nanoparticles are explored to be efficient intracellular transport systems, which provides retention of transfection molecules accessible to biological systems for *in vitro* and *in vivo* gene transcriptional studies addressing high efficacy in their roles in therapeutic perspective (Müller et al. 2000; Yu et al. 2016). Research evidence lipofectamine CRISPRMAX to be highly recommendable transfection agent considering cell toxicities exerted by the system, compared to lipofectamine 3000 and lipofectamine RNA Imax (Yu et al. 2016). The *in vitro* transfection efficacy of these unique elements varies depending on the temperature fluctuations, studies interpret reduced particle segregation during frozen state, enhancing a more pronounced transfection activity of these lipid nano - systems (Sork et al. 2016). Considering toxicities exerted by other delivery entities, studies showcase lipofectamine 2000 less toxic towards hepatic organs, moreover, their efficacy in delivering fluorescent tagged biological systems across intracellular destinations designate them attractive transfection system not only for *in vitro* experimental setting, but also for *in vivo* gene transfers as well (Gao et al. 2012; Enlund et al. 2014).

1.6.6 Gene therapy

Considering the after effects produced by conventional chemotherapy and radiation therapies and toxicities addressed by nano delivery systems, gene therapy is a promising platform for restoring the function of impaired target genes or enhancing the host defence mechanism, targeting only cancerous cells (Amer. 2014).

Gene therapy is a therapeutic strategy in treating conditions evolved due to gene abnormalities or mutations. The first successful effort of administrating target genes in cancer treatment was established by inserting tumour suppressor gene p53, in 2003 by Chinese

researchers (Pearson et al. 2004). Studies are exploring the efficiency of several biological delivery agents including viruses, nanoparticles, plasmid DNA and lipoproteins and non - biological methods including electroporation for safe and targeted gene delivery applications (Niidome. 2002). A novel biological transfection moiety referred to as TEMTAC (Tunable endogenous mammalian target complementation system) has been recently explored to procure multifunctional properties, involving switching on and off several transcriptional factors respective to normal cellular requirements in mammalian tissues (Benisty et al. 2015). Hence introducing these multifunctional implants may pave way for highly targeted and more promising treatment strategy, not only for cancers but also several other disorders associated with gene overexpression or in non - functional modes.

Latest investigation in gene therapy referred to as CAR T, which entitles the genetic modification of patient's immune cells *ex vivo* and reinstallation, act as powerful tool for the treatment of acute lymphoblastic leukaemia cases however, this therapeutic strategy is studied to be ineffective for solid malignancies (Richard et al. 2017).

In *in vitro* experimental settings, nucleic acids need to be safely transported across cellular membranes for successful gene transfection and expression studies, making experimental studies quite challenging. However, in clinical setting, although nucleic acids are studied to be easily administered through intravesical or intra - cutaneous routes for *in vivo* therapeutic applications, the treatment reflects reduced efficacy due to less genes reaching the desired destinations (Jinturkar et al. 2011). Hence to reflect a complete gene therapeutic index, the therapeutic genes needed to be successfully delivered to target destinations.

The second phase of this thesis have explored the cytotoxicity of chitosan and BSA based microgels and microparticles in colorectal cancer cell lines HT - 29 with the objective of using it for delivery of plasmid DNA for evaluation of transfection studies under non - cytotoxic circumstance.

1.7 Rationale of the study

Colorectal cancer is the world's third leading cancer according to recent statistics (Siegel. et al. 2014). One of the eminent challenges, cancer patients facing is the developing resistance to conventional therapeutics (Axelrod. et al. 2017). Hence the necessity of developing highly

personalised treatment strategies are at top priority. *In vitro* studies have evidenced the over-expression of p21 in inducing apoptosis, demonstrating that p21 protein in its inactive stage has turned into its active form in response to the anti - cancer agents and is found to play a crucial role in arresting the cells at the G1 and G2 phase of the cell cycle, thereby preventing cellular proliferation (Gartel. 2002; Pavelic et al. 2008). p21 gene functions a negative attenuator of DNA replication, in response to translation of several genes including PCNA and p53 in attenuating cancer proliferation (Pavelic et al. 2008).

Besides the tumour suppressor protein p21, another protein - HSPA1A is often elevated in tumour cells and has an anti-apoptotic activity. Eukaryotic cancer cells under physiological stress response are encountered with elevated levels of stress protein HSPA1A, which are studied to participate closely with signalling pathways assisting a protective role under cellular stress, enhancing tumorigenicity. Recent studies, reveals the resistance acquired by chemotherapeutic in colorectal cancer cells abstaining elevated levels of HSPA1A, expressing its defensive role against apoptosis (Grivicich. et al. 2007).

Hence, repression of HSPA1A proteins evidenced to improve the chemotherapeutic sensitivity in colorectal cancers (Sherman. 2015; Jagadish. et al. 2016). This thesis at the initial stage, investigated the inhibitory effects of HSPA1A using pifithrin - μ independently and in combination with UV and their roles in hyperthermia treatments in both leukaemia cancer cell lines U937 and colorectal cancer cell lines HT-29 to perceive the roles of HSPA1A in different cancer cells.

1.8 Objectives and hypotheses of the thesis

This thesis aimed at investigating whether a two - pronged approach, overexpressing p21 and inhibiting HSPA1A protein activity with pifithrin - μ, would be effective in inducing apoptosis in colorectal cancer cell line HT-29.

Considering, the main aim of research, the thesis had the following objectives and hypotheses:

1.To evaluate the effect of antimycotic/ antibiotic on colorectal cancer cell lines HT-29.

Hypotheses-

H0 Antimycotic/ antibiotic will not induce stress or inhibit the growth of HT29 cells.

H1 Antimycotic/antibiotic may induce stress response and inhibit the growth of HT-29 cells.

2.To investigate the role of HSPA1A protein in combination treatments including UV radiation or hyperthermia.

Hypotheses-

H0 UV radiation, pifithrin chloride, pifithrin - μ or hyperthermia at 42℃ treatment will not induce cell death in leukaemia cell lines U937 and colorectal cancer cell lines HT-29.

H1 UV radiation, pifithrin chloride, pifithrin - μ or hyperthermia at 42℃ will induce cell death in U937 and HT-29 and the inhibitory activity of HSPA1A protein by pifithrin - μ in combination with UV may enhance the treatment effect of UV in HT-29 and U937 cancer cell lines.

3.The effectiveness of chitosan or BSA modulated intracellular delivery systems for the successful and safe delivery of human p21 - plasmid DNA for transfection studies on colorectal cancer cell lines HT-29.

Hypotheses -

H0 chitosan or BSA modulated intracellular delivery systems will be non - cytotoxic in drug free states on HT-29 cells.

H1 chitosan or BSA modulated intracellular delivery systems will be cytotoxic in drug free states on HT-29 cells.

4. Whether the inhibitory activity of HSPA1A proteins would accelerate the apoptosis induced by p21 plasmid DNA transfection in HT-29 cell lines.

Hypotheses -

H0 transfection with p21 plasmid DNA will not arrest the growth of HT-29 cells and inhibition of HSPA1A in combination with increased p21 will not enhance apoptosis in HT-29 cells.

H1 transfection with p21 plasmid DNA may arrest the growth of HT-29 cells and the inhibition of HSPA1A in combination with increased p21 will enhance apoptosis in HT-29 cells.

Chapter 2

2.1 Materials

Table 2.1.1 Laboratory materials

Consumables	Manufacturer	Catalogue number
Micropipette (1-5 ml)	Thermofisher scientific	LH 96952
Micropipette (1000 µl)	Thermofisher scientific	MH 60159
Micropipette (100-500 µl)	Thermofisher scientific	MH 59984
Micropipette (10 -100 µl)	Thermofisher scientific	MH 37243
Micropipette (1 - 10 µl)	Thermofisher scientific	MH 59944
Micropipette (0.2 - 2 µl)	Thermofisher scientific	MH 59204
Microplate 96-well sterile flat bottom lidded	Costar	01317002
Microplate 96-well sterile v- bottom lidded	Costar	CUS 326801
Microplate 6-well sterile flat bottom lidded	Thermo scientific	01617052
Tissue culture flasks (25cm^3)	Costar	136196
Centrifuge tubes (15 and 50 ml)	Thermo scientific	156499
Micro centrifuge tubes (0.5, 1.5 and 3 ml)	Thermo scientific	S1615,5500
Micro adhesive plate seals	Thermo scientific	00445503

Table 2.1.2 Laboratory equipments

Equipment	Manufacturer	Catalogue number
Incubator (water jacketed)	Thermo scientific	20150709
Biological safety cabinet	Thermo scientific	41852324
Microscope	EVOS, Life technologies	SN 10415-1727-371
Haemocytometer	EVOS, Life technologies	B 1015-181C-024
Cooling centrifuge (Heraeus, multifugeX3R)	Thermo scientific	75004515
Centrifuge (Heraeus, Fresco 17)	Thermo scientific	75002420
Mini vortexer	Thermo scientific	14-955-151
Water bath	Fisher brand	115724
Hot plate vortexer	Clifton	01270524871
Microplate reader (Varioskan LUX)	Biocote	3020166
pH meter (AB 150Ph/mv	Thermo scientific	13-620-631
Nano drop (Simplinano)	Fisher scientific	29061712
BD Accuri flow cytometer	Biochrome LTD	-
Electrophoresis system	BD Biosciences	-
Power pack (1000 volt)	Thermo scientific	150728116
G-Box gel imaging (Chemi X RQ)	Syngene	D 24V2/3345
Scanning electron microscope	Gemini	-

Table 2.1.3 Materials for cell culture and viability measurement assays

Consumables	Manufacturer	Catalogue number
Leukaemia cell line -U937	European culture collection	85011440
Colorectal cancer cell line- HT-29	European culture collection	91072201
Eagle's Minimal Essential Medium (EMEM)	Lonza	BE 12-611F
Roswell Park Memorial Institute (RPMI)1640	Lonza	BE 12-702F
Foetal bovine serum (FBS)	Life technologies	10270-106
Trypsin-EDTA	Lonza	BE 17-161E
Dimethyl sulfoxide (DMSO)	Sigma aldrich	67-68-5
Trypan Blue	Sigma aldrich	T 8154
MTS reagent (4,5-dimethylthiazol-2yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium	Promega	G 3 582 SC 215699
Phenazine ethosulphate (PES)	Santa Cruz	P4170
Antimycotic/ antibiotic solution	Sigma aldrich	A 1593

Table 2.1.4 Chemotherapeutic Drugs

Consumables	Manufacturer	Catalogue number
Pifithrin - μ	Sigma aldrich	P 0122
Pifithrin chloride	Calbiochem	53106
5 - Fluorouracil	Sigma aldrich	F 6627

Table 2.1.5 Reagents for microgel/microparticle preparation

Consumables	Manufacturer	Catalogue number
Low molecular weight chitosan powder	Sigma aldrich	448869
β - glycerol phosphate	Sigma aldrich	G 9891-25G
Glutaraldehyde	Sigma aldrich	G 5882
Sodium tripolyphosphate	Sigma aldrich	238503
Bovine serum albumin powder	Fisher Scientific	E 0665 DF/17

Table 2.1.6 Reagents for flow cytometer

Consumables	Manufacturer	Catalogue number
BD Cytofix/cytoperm	B D Biosciences	51-2090KZ
FITC Annexin V	BD Pharmingen	556419
Propidium iodide	Sigma aldrich	25535-16-4
DPBS -Dulbecco's phosphate buffered saline (500 ml)	Lonza	BE 17 513F

Table 2.1.7 Antibodies consumed

Antibodies	Manufacturer	Catalogue number
p21 Antibody (FITC conjugated)	Santa cruz biotechnology	SC 6246
HSPA1A Primary antibody DEG	Purified in Laboratory	
HSPA1A Secondary antibody (extravidin FITC)	Sigma aldrich	E 2761

Table 2.1.8 Reagents for bacteriological media preparation

Consumables	Manufacturer	Catalogue number
Magnesium chloride	Sigma aldrich	M – 1028
Magnesium sulphate solution	Sigma aldrich	M - 3409
Lactose	Sigma aldrich	63 – 42 - 3
Tryptone	Lab M Limited	M C005
Yeast extract	Lab M Limited	M C 001
Agar	Oxoid	L P 0013
Glucose	Sigma aldrich	59 - 99 - 7
Sodium chloride	Fisher scientific	7647 - 14 - 5
Ampicillin	Fisher scientific	11668030

Table 2.1.9 Commercial kits for p21 plasmid DNA based studies

Commercial Kit	Manufacturer	Catalogue number
Flag p21 plasmid	Addgene	16240
Endofree maxi prep kit	Qiagen	12162
Restriction enzyme BamHI (10 U/ µl)	Thermo scientific	ER0051
Restriction enzyme Ndel (10U/µl)	Thermo scientific	Er0581
Hyper ladder (100 lanes)	Bioline	BIO-33053
Gel red stain	Biotium	41003
Agarose gel	Promega	V3121
TAE buffer (50X)	Fisher bioreagent	BP1332-1
Isolate II PCR and gel kit	Bioline	BIO 52058
DNA repair kit	Thermofisher scientific	IVGN2504
Pure link PCR purification kit	Invitrogen	K3100-01
Quick stick ligase kit	Bioline	BIO 27027
DH5 α chemical competent cells	Bioline	BIO-85026
Lipofectamine 2000	Invitrogen	11668 - 030

2.2 Preparation of tissue culture medium, reagents and buffers

2.2.1 Tissue culture medium

Eagle's Minimal Essential Medium (EMEM) and Roswell Park Memorial Institute Medium (RPMI)1640 were supplemented with 10% of foetal bovine serum. The medium was stored at -20℃ and thawed in water bath preset at 37℃ before use.

2.2.2 Reagents for cell - based assays

2.2.2.1 MTS reagent

MTS reagent 0.20% w/v was prepared using DPBS solution using magnetic stirrer. 1M HCl was used to adjust the pH of the solution to 6.5. Phenazine ethosulphate (PES) (0.09% w/v) was prepared in DPBS solution. The pre - prepared MTS solution 40 ml was incorporated with 2 ml of PES thus prepared. The solution was aliquoted and covered for protection from light and stored for future use at -20℃.

2.2.2.2 Phosphate buffered saline (PBS)

1L of PBS was prepared by using sodium chloride (0.8% w/v), potassium chloride (0.02% w/v), potassium dihydrogen phosphate (0.024% w/v) and sodium phosphate (0.144% w/v). The solutes were dissolved in 850 ml of distilled water using magnetic stirrer. Upon complete dissolution of the solutes, the solution was made up to 1L with distilled water.

2.2.2.3 PI reagent

PI reagent (0.01% w/v) was prepared using PBS solution. The reagent was aliquoted and protected from light and stored at -20℃.

2.2.2.4 Blocking buffer 10 ml

Blocking buffer was prepared with FBS (5% v/v) in DPBS solution. Freshly prepared blocking buffer was used for experiments.

2.2.3 Preparation of chemotherapeutics

2.2.3.1 Pifithrin chloride (1mM)

1mM working solution of Pifithrin chloride was prepared by solubilizing 10 µl of (100 mM) stock in 990 µl of culture medium.

2.2.3.2 Pifithrin - µ (100 mM)

551.84 µl of filter sterilized DMSO was added to 10mg of pifithrin - µ to make 100 mM stock solution.

2.2.3.3 5 - Fluorouracil (100 mM)

10mg of 5 - Fluorouracil was dissolved in 768.75 µl of filter sterilized DMSO solution to make 100 mM stock solution.

2.2.4 Reagents for chitosan and BSA based microgels and microparticles

2.2.4.1 Chitosan solution (1% w/v)

Chitosan solution (1% w/v) was prepared using Low molecular weight chitosan in HCL (0.1 M) solution by magnetic stirring. The solution was filter sterilized prior to use.

2.2.4.2 β - glycerol phosphate solution

1ml β - glycerol phosphate solution of (0.5, 1, 5, 10% w/v) concentrations, were prepared by dissolving (0.005, 0.01, 0.05, 0.1g) beta glycerol phosphate powder accordingly using exactly 1ml of distilled water. The mixture was sterile filtered before use.

2.2.4.3 Hydrochloric acid (HCL) 10 ml (0.1M)

10 ml of 0.1M HCL was prepared by diluting 1 ml of 1M HCL solution in a standard flask and make this up to 10 ml using distilled water and filter sterilized.

2.2.4.4 Sodium tri - poly phosphate 10 ml

Sodium tri - polyphosphate (0.1% w/v) was prepared using distilled and filter sterilized.

2.2.4.5 Aqueous acetic acid 10 ml (1% v/v)

Acetic acid (1% v/v) was prepared by suspending 0.1ml of 99.9% acetic acid into 10 ml standard flask. The solution was made up to 10 ml using distilled water.

2.2.4.6 BSA (10% w/v)

0.1 g of BSA was suspended in 1 ml of pre - prepared 10mM NaCl solution to obtain 10% w/v concentration of BSA.

2.2.4.7 Sodium chloride (10mM)

0.00584 g of sodium chloride was dissolved in 10 ml of distilled water to make a stock of 10mM.

2.2.5 Reagents for agarose gel electrophoresis

2.2.5.1 Agarose gel

Agarose gel (0.7% w/v) was prepared by dissolving 0.24g of agarose powder in 35 ml of pre-prepared 1X TAE buffer, using hot plate magnetic stirrer pre - set above 100℃. The mixture was observed for complete dissolution. The mixture could cool as the hot solution may warp the casting apparatus. Upon reaching bearable temperature, 3.5 µl of nucleic stain gel red was added to the agarose. The gel solution was subsequently incorporated into the pre - casted gel apparatus and allowed to cool for 45 - 60 min until it is set for experimental proceedings.

2.2.5.2 TAE buffer (1X)

1 litre of (1X) TAE buffer was prepared by adding 20 ml of (50X) TAE stock solution into a standard flask and make up to exactly 1 L using distilled water.

2.2.5.3 EDTA 1 ml (0.5 M)

0.14612 g of EDTA was dissolved in 1 ml of distilled water to make 0.5 M EDTA solution.

2.2.6 Preparation of reagents and microbiological growth media

2.2.6.1 Ampicillin antibiotic 10 ml (1% w/v)

0.1g of ampicillin antibiotic was dissolved in 10 ml distilled water to obtain a final concentration of 1% w/v. This was filter sterilized before use.

2.2.6.2 Preparation of Luria-Bertani broth

Sodium chloride(4g), Tryptone (4g) and 2g of yeast extract were weighed into 360 ml of distilled water in a clean glass bottle. The mixture was dissolved using magnetic stirrer. The pH of the solution was then calibrated to 7.0 using 5M NaOH, and distilled water was used to make up the total solution volume to 400 ml. This was subjected to autoclaving. Following autoclaving, the broth was cooled, and filter sterilized ampicillin antibiotic (1% v/v) was added prior to use.

2.2.6.3 Preparation of Luria-Bertani agar

Sodium chloride (4g), Tryptone (4g), 2g of yeast extract and 8g of agar were weighed into 360 ml of distilled water in a clean glass bottle. The added solutes were dissolved with the aid of magnetic stirrer. The pH of the solution was then calibrated to 7.0 using 5M NaOH, and distilled water was used to make up the total solution volume to 400 ml. Following autoclaving, the mixture was cooled and incorporated with filter sterilized ampicillin antibiotic (1% v/v). The mixture was uniformly poured in 20 ml volume into sterile disposable petri plates and allowed to solidify. The agar plates were stored in refrigerator at 4°C with the lid upside down position or directly used.

2.2.6.4 Preparation of SOC medium

Sodium chloride (0.1g), Tryptone (4g) and Yeast extract (1g) were weighed into 150 ml of distilled water in a clean glass bottle. The added solutes were dissolved using magnetic stirrer. The mixture was made up to 200 ml using distilled water, autoclaved and allowed to cool. The medium was incorporated with filter sterilized 1M (1% v/v) of Magnesium chloride and magnesium sulphate. The medium was incorporated with 4 ml of 20% v/v sterile glucose prior to experimental use.

2.3 Methods

2.3.1 Tissue culture

2.3.1.1 U937 Leukaemia cancer cell line

The leukaemia cancer cell line U937 in suspension were maintained in RPMI culture medium pre - supplemented with 10% foetal bovine serum. The cells were cultured in 25cm^2 tissue culture flask under sterile cell culture conditions and incubated at 37℃ in the incubator provided with 5% CO_2 and 95% air. The cells were observed every 24 h for normal growth. The cells upon reaching confluency of 70 - 75%, were sub cultured by seeding 1 ml of cells in 9 ml of culture medium.

2.3.1.2 Colorectal cancer cell line HT - 29

Adherent cell lines of colorectal cancer HT-29 were maintained in EMEM cell culture medium under standard cell culture conditions in 25cm^2 or 75cm^2 cell culture flask in incubator provided with 5% CO_2 and 95% air at 37℃. The attached cells were subcultured upon reaching confluency of 70 - 75%, every 72 h. The cells were rinsed with 1 ml of trypsin/EDTA (0.25%) and the cells were resuspended with 2.5 ml of trypsin/EDTA (0.25%) and incubated for 5 - 7 min in incubator at 37℃. The cells were then microscopically observed for surface detachment. Trypsin was deactivated by addition of 7.5 ml of EMEM medium incorporated with 10% FBS - containing α -1 antitrypsin, which inhibits the protease activity of trypsin. The cells were then pooled out from the culture flask using sterile pipettes onto sterile 15 ml centrifuge tubes. The cells were centrifuged at 500 x *g* for 5 min. The cells were then resuspended in EMEM culture medium and subcultured by seeding 2 ml of cell culture in 8 ml of culture medium.

2.3.2 Frozen cell Storage and recovery

The colorectal cancer cell line HT-29 and leukaemia cell line U937 were subjected to cryopreservation for future use. The U937 cells in suspension upon reaching growth confluency of 50 - 60% in cell culture medium were extracted by centrifugation at 500 × *g* for 5 min. The cells were then resuspended in 1 ml of freezing medium comprising of 10% of DMSO in foetal bovine serum in pre - labelled 1 ml cryo vials. The HT-29 cell lines were

extracted as illustrated in (section 2.3.1.2), centrifugated at 500 × *g* for 5 min. The cells were then resuspended in 1 ml of freezing medium comprising of 10% of DMSO in foetal bovine serum in pre - labelled 1 ml cryo vials. The cryovials following 2 h incubation in liquid nitrogen were subsequently transferred into the cryostat.

2.3.2.1 Recovery procedure

The cancer cell lines stored in 1 ml cryovials in the cryostat were removed and recovered by thawing in a water bath pre - set at 37℃. The cells were then transferred into 25 cm² tissue culture flask containing 9 ml of cell culture medium. The cells were then incubated in incubator provided with standard tissue culture conditions.

2.3.3 Determination of cell viability by microscope

The cell viability of both the cancer cell lines were determined using trypan blue exclusion method prior to all experiments performed. The cells in suspension state (100 µl) were suspended in trypan blue reagent (100 µl) and 20 µl of cells in trypan blue was placed in haemocytometer and sealed using sterile cover glass. The cells were then observed and assessed for viability using light microscope. The viable cells were observed white in colour and dead cells due to disruption in cell membrane, absorbed the trypan blue dye and appeared blue in colour.

2.3.4 MTS cell viability assay

The MTS cell viability assay is based on enzymatic reaction. The compound tetrazolium present in MTS reagent is catalysed into a chemical known as formazan due to the presence of NAD (P) H-dependent dehydrogenase in viable cells. The cell viability is assessed at 490nm using plate reader. The MTS assay absorbance values defines the cell viability from the quantity of the reduced compound in the cell pool.

The U937 cancer cell lines in suspension in 25 cm² tissue culture flask was counted using a haemocytometer. The colorectal adherent cancer cell lines grown in 25 cm² was trypsinated using trypsin EDTA as illustrated in (section 2.3.1.2). The cells count was determined using haemocytometer (section 2.3.3). The 96 well sterile assay plates were suspended with 100 µl/well of cancer cells at cell density of approximately 5 × 10^5 cells/ml. Untreated cell lines were placed as controls. Dead cells plated at the same density were prepared by repeated

cycles of simultaneous freezing in liquid nitrogen and thawing in water bath pre - set at 37℃. Culture medium alone was also placed as reference. Treatment with U937 cancer cell lines were performed simultaneously upon seeding the cells in 96 well assay plates. However, the colorectal cancer cell lines HT-29 seeded onto the 96 well assay plates were allowed for attachment for 24 h at 37℃ in incubator. The cells upon attachment were replaced with fresh medium, except for the dead controls to prevent cell loss and subsequent treatments were performed. The cells were then allowed for incubation for 24 h or 48 h for the treatments. The cells following subsequent incubation are measured for cell viability by MTS assay. Approximately 20 µl of the pre - prepared MTS working reagent (section2.2.2.1) was added to each well of the 96 well assay plates containing cells and allowed for incubation for 1 - 2 hours providing 37℃ in incubator. The cell viability was determined by measuring the quantity of the reduced product at an absorbance of 490nm using plate reader.

2.3.5 Propidium iodide assay

The propidium iodide assay is used to determine cellular necrosis. The intercalating bases of the DNA of the necrotic cells due to the absence of membrane stability are subsequently permeable to this fluorescent dye. The cells in cell culture medium, upon subsequent treatments were subjected to treatment with propidium iodide (0.5% v/v) working dilution in sterile phosphate buffered saline (section 2.2.2.2)**.**

Both the cancer cell lines used in the experiments were counted using haemocytometer (section 2.3.3). The cancer cells 100 ul/well at cell density of approximately of 5×10^5 cells/ml, were seeded onto 96 well sterile assay plates. Untreated cell lines were placed as controls. The dead cell controls were prepared by repeated cycles of simultaneous freezing in liquid nitrogen and thawing in water bath pre - set at 37℃. Culture medium alone was also placed as reference. Treatment with U937 cancer cell lines were performed simultaneously upon seeding the cells in 96 well assay plates. However, the colorectal cancer cell lines HT-29 were seeded onto the 96 well assay plates were allowed for attachment for 24 h at 37℃ in incubator. The cells upon attachment were replaced with fresh medium, except for the dead controls to prevent cell loss and subsequent treatments were performed. The cells were then allowed for incubation for 24 h or 48 h for the treatments. Following incubation, the cells in culture medium were incorporated with 100 µl/well of propidium iodide dye, the cells were

subjected to incubation at 18℃ provided protection from light for 20 min. The relative fluorescence thus produced were measured using microplate reader (Varioskan LUX).

2.3.6 Determination of annexin V and PI by flow cytometer

The cancer cell lines U937 and HT-29 were detached from 75 cm^2 tissue culture flasks using trypsin/EDTA as illustrated in (section 2.3.1). The cell count was determined using haemocytometer (section 2.3.3). Cells at cell density of 1×10^6 cells/ml were seeded onto each well of the sterile 6 - well assay plates. HT-29 cell lines were allowed for attachment for 24 h incubation at 37℃. U937 cell lines were subjected to subsequent treatments simultaneously upon cell seeding onto 6 well assay plates. Following cell treatments and incubations, the cells were detached using trypsin/EDTA and transferred onto sterile v - bottom 96 well assay plates for cell preparation for flowcytometric assay. The cells were subjected to centrifugation in centrifuge pre - set at 500 x *g* for 5 min. The cells were then resuspended with ice cold phosphate buffered saline solution (100 µl) and centrifuged. This washing procedure was performed for 3 consecutive times. Following subsequent washing steps, all the treated cells, except for the controls were suspended with equal volumes of annexin V and PI reagents (2.5 µl each) diluted in 50 µl of binding buffer (1x). The annexin V reference were treated with only annexin V reagent (2.5 µl in 1X binding buffer) and PI reference control with PI 2.5 µl in 1X binding buffer). The assay plates were placed in plate shaker for uniform mixing of the reagents and placed in the dark for 15 min. All the cells were then subjected to addition with 200 µl of 1x binding buffer and flow cytometric analysis was performed within 60 min.

2.3.7 Flow cytometric determination of HSPA1A and p21 protein

2.3.7.1 Evaluation of intracellular and cell surface HSPA1A protein

The cancer cell lines at cell density of 1×10^6 cells/ml /well of the 6-well assay plates, upon subsequent treatments were centrifuged at 500 x *g* for 5 min and resuspended in 100 µl of cell culture medium and transferred into 96 well v - bottom assay plates for preparing the cells for flowcytometric determination of protein expression. The cells were then centrifuged at 500 x *g* for 5 min and resuspended uniformly in 200 µl of DPBS solution to prevent formation of cell clumps. This washing procedure with DPBS was repeated for three

consecutive intervals. For determining intracellular proteins, the cells following centrifugation were resuspended in 70 µl of cell fixative and permeating reagent, sealed and placed in refrigerate for 20 min. Followed by 20 min refrigeration, the cells in fixative were diluted by adding 70 µl of DPBS solution without mixing. The cells were then centrifuged at 500 x *g* for 5min and resuspended with 100 µl of blocking buffer for 5min and again centrifuged.

The cells were resuspended with primary HSPA1A antibody in the ratio 1:100 (antibody: FBS blocking buffer- 5% v/v in DPBS) and allowed for incubation for 10 min. Following incubation, the cells were centrifuged and resuspended in blocking buffer (FBS 5% v/v in DPBS) for 5 min duration and the cells were resuspended with 50 µl of extravidin FITC labelled secondary antibody in the ratio 1:200 and allowed for incubation for 10 min. The cells were then incorporated with 50 µl of blocking buffer (FBS 5% v/v in DPBS) and centrifuged. And finally, the cells were resuspended in 100 µl of DPBS solution and analysed using flow cytometer.

2.3.7.2 Evaluation of extracellular HSPA1A proteins

For determination of extracellular HSPA1A proteins by flow cytometer, the cancer cells upon treatment with antimycotic/ antibiotics, pifithrin - µ or hyperthermia were subjected to all the steps described in (section 2.3.7.1), except that of incorporation of cell fixative and permeating reagent as this may permeabilize the cell membrane, which could result in antibodies to detect HSPA1A in the cell interior.

2.3.7.3 Flow cytometric determination of p21 proteins

Colorectal cancer cell lines HT-29 subjected to p21 plasmid DNA transfection studies (section 2.3.21) were evaluated for p21 protein expression at both the intracellular and cell surface levels using FITC conjugated p21 primary antibody using flow cytometer.

2.3.7.4. Evaluation of intracellular p21 proteins

Colorectal cancer cell lines HT-29 were seeded at cell density 1×10^6 cells in 6 well assay plates, the cells could attach for 24 h in EMEM medium devoid of FBS in incubator at 37℃, provided with standard cell culture conditions. Following incubation, the cells were transfected with p21 plasmid DNA and pMT5 empty vector using transfection reagent lipofectamine 2000 and EMEM medium without FBS. Untreated cells were placed as controls. The transfection was allowed for 24 h. The untreated cells, cells resulted from transfections

were detached using trypsin EDTA as illustrated in (section 2.3.1.2) and floating cells were collected and centrifuged at 500 x *g* for 5 min. The cells were then resuspended in EMEM medium and transferred onto 96 well - v bottom assay plates. The cells were subjected to centrifugation, for 5 min, followed by resuspension in 200 µl cold DPBS. The cells following centrifugation, were resuspended in 70 µl cell fixative. The cells were then placed under sealed condition in refrigerator for 20 min. Following refrigeration, the fixative was diluted with the addition of DPBS (70 µl). This was followed by resuspension in DPBS (100 µl), centrifuged and resuspended with pre - prepared blocking buffer (100 µl) (section 2.2.2.4). Following incubation with blocking buffer (FBS 5% v/v in DPBS) for 5 min at room temperature, centrifugation was performed, and cell were resuspended with 1µg (10 µl) of FITC labelled p21 antibody/1 × 10^6 cells in 50 µl of blocking buffer (FBS 5% v/v in DPBS). The antibody was uniformly mixed, and the cells were sealed, covered to protect from light and placed in refrigerator for 45 min. The cells were then incorporated with blocking buffer 50 µl and subjected to centrifugation for 5min at 500 x *g*. The cells were then finally resuspended with 100 µl of DPBS solution and measured by flow cytometer.

2.3.7.5 Evaluation of cell surface p21 proteins

For determination of cell surface p21 proteins by flow cytometer, the cancer cells upon subsequent treatments were subjected to all the steps described in (section 2.3.7.4), except the addition of fixative and 20 min of refrigeration, followed by dilution of fixative with DPBS solution, which was excluded as the fixative may permeabilize the cell membrane, which could result in antibodies to detect p21 in the cell interior.

2.3.8 Preparation of chitosan microgels

Chitosan microgels and microparticle delivery systems were prepared to analyse the effect of these agents independently and in combination with chemotherapeutic 5 - fluorouracil in colorectal cancer cell lines HT-29, with the objective of using it for the safe delivery of p21 plasmid DNA in HT-29 cell lines.

Chitosan microgels were prepared by simultaneous drop wise addition of 15℃ β - glycerol phosphate solution (concentrations ranging – 10%, 5%, 1% and 0.5%) (section 2.2.4.2) into 15℃ low molecular weight chitosan (1% w/v) section 2.2.4.1) in 0.1M hydrochloric acid

solution (section 2.2.4.3) under continuous vortexing. 5 - fluorouracil was delivered using chitosan microgels for enhanced cytotoxic activity. The 5 - fluorouracil at varying concentrations of 5 and 10 µM were mixed in 500 µl of cold chitosan solution separately. 125 µl of cold β - glycerol phosphate solution was added dropwise to the cold 5 fluorouracil - chitosan mixtures with continuous vortexing. The pH of the solution was adjusted to 7.0 using 1M sodium hydroxide solution. All the solutions were passed through sterile membrane filters. The chitosan - β glycerol phosphate emulsion thus formed by vortexing was placed in water bath pre - set at 37°C for 3 - 4 h until the microgels were formed (Fig 2.1).

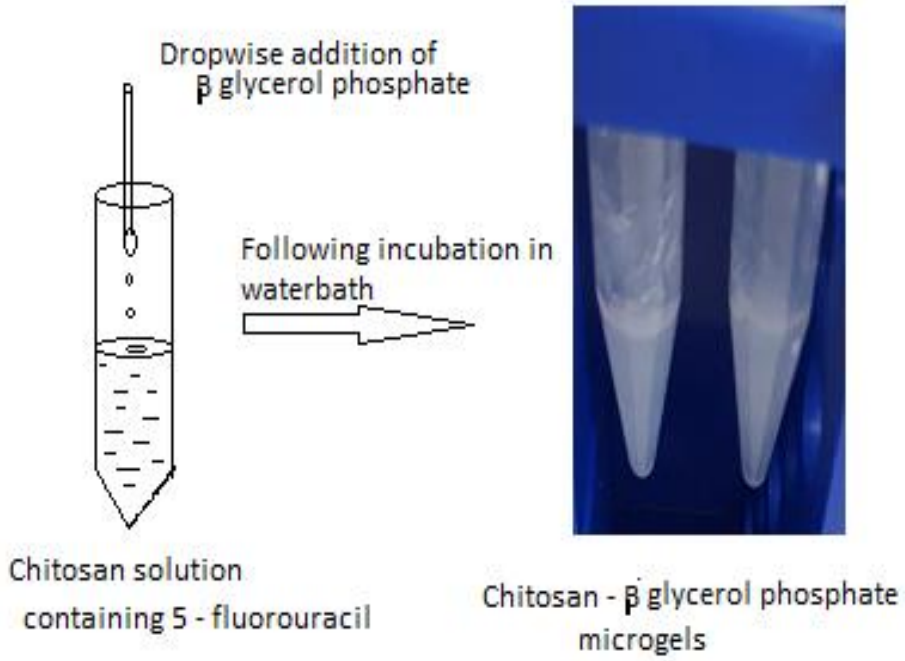


Fig 2.1 Preparation of chitosan - β - glycerol phosphate modified microgel systems.

2.3.9 Preparation of chitosan microparticles

Chitosan microparticles were prepared by modifying the ionic gelation method (Fig 2.2) by adjusting the pH (7.0). The low molecular weight chitosan powder 0.5% w/v was prepared using aqueous acetic acid (1% w/v) solution by magnetic stirring. The microparticles were formed simultaneously by dropwise addition of chitosan (2.5 ml) into 1ml of (0.1%) cross linking agent sodium tri - polyphosphate (TPP) pre - incorporated with glutaraldehyde (20%) under strong magnetic stirring for 3 - 4 h. 5 - fluorouracil particle combinations were produced by mixing 5 - fluorouracil in chitosan solution prior to addition of the cross - linking agent. The

particles thus formed were centrifuged at 15,000 x *g* for 30 min. The particles were washed using distilled water and centrifuged 15,000 x *g* for 30 min. Followed by centrifugation, the particles were resuspended in distilled water, sterile filtered and added to HT-29 cell lines pre-seeded in 96 well assay plates in EMEM medium containing 10% FBS. 5 - fluorouracil free microparticles, 5 - fluorouracil treated, untreated cells and dead cells were placed as controls.

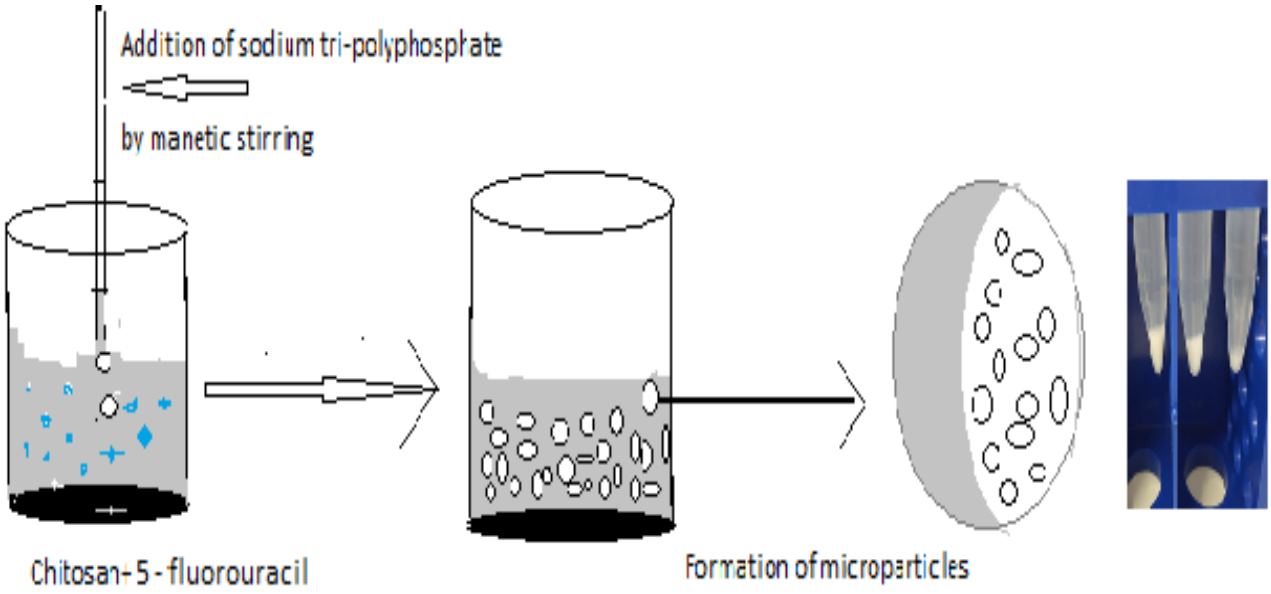


Fig 2.2 Preparation of Chitosan - TPP -5 - fluorouracil microparticles.

2.3.10 Preparation of bovine serum albumin microparticles

Bovine serum albumin protein microparticles were prepared to analyse its effect independently and in combination with chemotherapeutic 5 - fluorouracil in colorectal cancer cell lines HT-29, with the objective of using it for the safe delivery of p21 plasmid DNA in HT-29 cell lines.

BSA microparticles were prepared by ethanol dissolvation - temperature induced denaturation method (Yu et al. 2014). The BSA in powder form was dissolved in NaCl solution (section 2.2.4.7) to obtain a final concentration of 10% w/v solution and filter sterilized. 1 ml of bovine serum albumin particles pre - incorporated with 5 fluorouracil were subjected to

dropwise incorporation of approximately 10 ml of ethanol at the rate of (1ml/min) to BSA suspension under magnetic stirring. Ethanol was added until the BSA solution turned turbid. The turbid BSA solution was then subjected to heat denaturation by transferring the emulsion containing tubes in water bath pre - set at 80 ℃ for 1 h. The microparticles thus formed were centrifuged at 15,000 x *g* for 30 min and subjected to washing using distilled water. Following centrifugation, the particles were resuspended in distilled water, sterile filtered and added to HT-29 cell lines pre-seeded in 96 well assay plates in EMEM medium containing 10% FBS. 5 - fluorouracil free microparticles, 5 - fluorouracil treated, untreated cells and dead cells were placed as controls.

2.3.11 Sample preparation for scanning electron microscopy

A drop of the sample to be scanned was mounted onto SEM sample stub fixed with a piece of double-sided carbon tape to create adhesive surface both to the stub surface and to the sample placed on the carbon tape. Compressed air was used to remove excess loose sample elements to ensure complete fixation. The samples were then observed by the scanning electron microscope.

2.3.12 Bacterial culturing and storage

2.3.12.1 Culturing

The full length p21 gene was a gift from Mien – Chie Hung, Addgene, United states. The *Escherichia coli* strains incorporated with p21 plasmid DNA was inoculated onto sterile Luria Bertani agar medium containing ampicillin antibiotic using streak plate technique. The plates were incubated with the lid facing down in incubator pre - set at 37℃ for 24 h. Bacterial starter cultures (500 ml) were then prepared by inoculating the bacterial inoculum in Luria Bertani broth pre - incorporated with ampicillin antibiotic. Following incubation at 37℃ for 24 h the broth culture was then ready for experimental proceedings.

2.3.12.2 Bacterial storage

The bacterial broth cultures for prolonged use were aliquoted into sterile eppendrof tubes 0.5 ml each. 0.5 ml of 50% v/v filter sterilized glycerol was added to each of the tubes containing bacterial broth cultures. The tubes were labelled and stored at -80℃**.**

2.3.13 Extraction of p21 plasmid DNA from bacterial cells

The plasmid DNA was extracted from the bacterial cells using Qiagen endofree maxi prep kit as illustrated as follows:

- *Escherichia coli* pure cultures carrying human p21 plasmid DNA were subcultured in 250 ml of lactose broth pre - incorporated with ampicillin antibiotic (1% w/v) in incubator at 37℃ for 24h.
- The bacterial cells were collected by centrifugation at 6000 x *g* using cooling centrifuge pre - set at 4℃ for 15 min.
- The pelleted cells were re - suspended in buffer P1 (10 ml) and then in 10 ml of buffer P2 with uniform mixing for 5 min at 25℃**.**
- Following incubation, the cells were mixed with buffer P3 (10 ml) and mixed until the suspension was clear.
- The bacterial lysate was subjected to filtration using QIA filter cartridge thus provided with the kit to remove chromosomal DNA, proteins or detergents from the bacterial lysate.
- Following filtration, the lysate was treated with 2.5 ml of buffer ER with continuous mixing and incubated for 30 min on ice.
- Following incubation, the lysate was then passed through a Qiagen tip column pre - equilibrated using buffer QBT thus provided within the kit, which allows successful binding of the plasmid DNA, along with removal of cellular proteins including degraded RNA and cellular metabolites in the lysate.
- The plasmid DNA retained within the Qiagen tip was purified by washing procedure using 6 ml of buffer QC.
- The plasmid DNA was finally eluted from the Qiagen tip using buffer QN 15ml into sterile tube free of endotoxin.
- The plasmid DNA was precipitated upon treatment with 10.5 ml of 70% v/v isopropanol and subjected to centrifugation in cooling centrifuge pre - set at 15,000 x *g* and 4 ℃ for 30 min duration.

- Following centrifugation, the supernatant was discarded, and the pellet thus retained was subjected to washing using 5 ml of 70% v/v ethanol thus provided. The pellet was then carefully retained following centrifugation at 15,000 x *g* for 10 min.
- The pellet following air drying was resuspended with required levels of buffer TE thus provided with the Qiagen kit.
- The DNA in TE buffer was then quantified using nano drop.

2.3.14 Sub cloning of the pMT5 vector DNA

The p21 plasmid DNA was subjected to restriction digestion to isolate the pMT5 vector DNA. The restricted Pmt5 vector DNA was separated using agarose gel electrophoresis and purified from the agarose gel, repaired for blunt ends, ligated, purified and sub cloned on to DH5 alpha competent *Escherichia coli* cells for its use as vector DNA control in p21-plasmid DNA transfection study in HT-29 cell lines as illustrated as follows:

2.3.15 Restriction of p21 gene and purification of PMT5 vector DNA

This was performed by optimization of Thermo scientific double restriction protocol thus illustrated as follows:

Tango buffer (1X)	4 µl
Restriction Enzyme BamHI (10U/ µl)	4 µl
Restriction Enzyme Ndel (10U/ µl)	4 µl
DNA (118.3 µg/ml)	5 µl (591.5 ng DNA)
Nuclease free water	3 µl
Total volume	**20ul**

The double digestion was proceeded by uniform mixing and allowed for incubation for 15 min at 37℃. Following incubation, the restriction enzyme activity was arrested using pre - prepared 0.5M EDTA to 20mM concentration of the total restriction mixture.

2.3.16 p21 plasmid DNA map

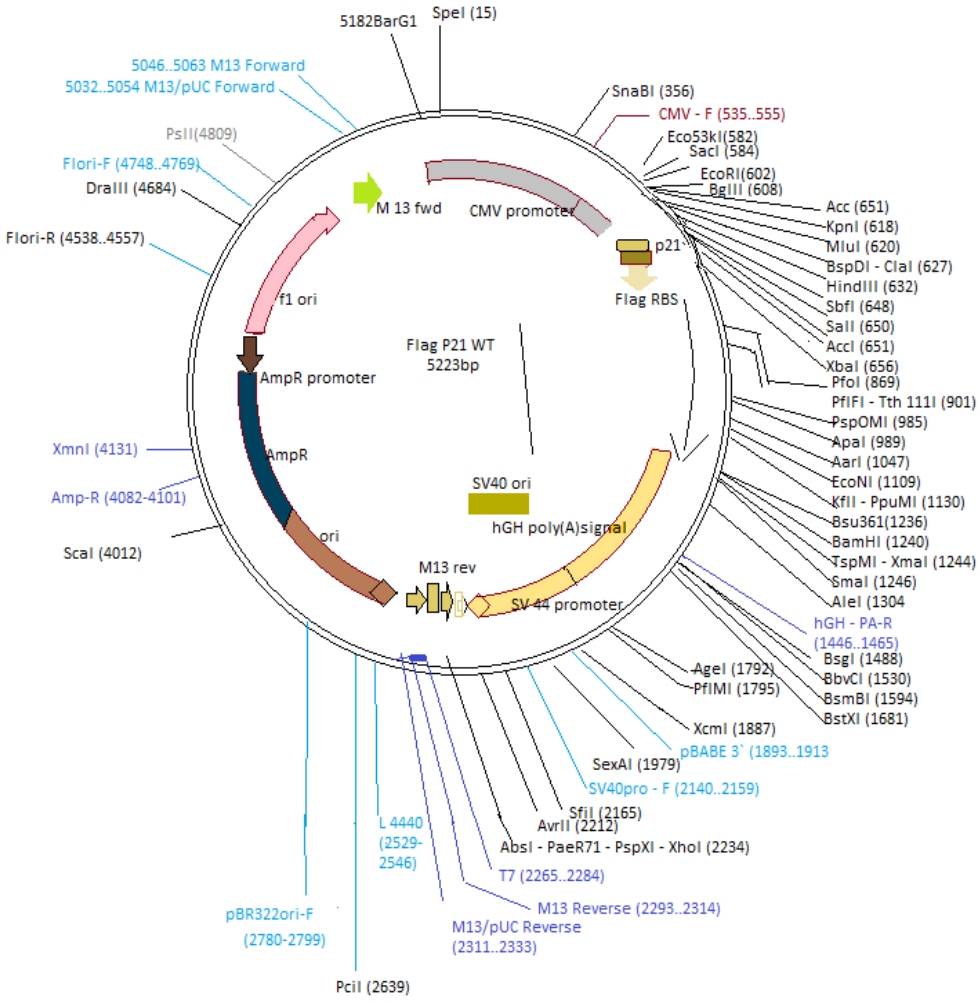


Fig 2.3 Illustrates the p21 plasmid DNA #16240 map adapted from the Addgene plasmid depository.

2.3.17 Electrophoretic separation of pMT5 vector

The restriction digestion mixture from (section 2.3.15) was subjected to agarose gel electrophoresis, for electrophoretic separation of restricted p21 plasmid DNA and pMT5 vector. The electrophoresis was performed as follows:

- 1% agarose gels in TAE buffer were loaded with hyper ladder (2.5 µl) and 10 µl of DNA sample (0.281 µg) diluted in 2 µl 6x loading dye, along undigested plasmid DNA. The gel concentration was optimized for obtaining separation of large kb DNA fragments.

- The gels were then subjected to electrophoresis at 100 volts for 50 - 60 min. Following sample running, the gels were carefully removed and analyzed by gene G - Box gel imaging (Chemi X RQ) software.

2.3.18 Extraction and purification of pMT5 vector from agarose gel

The pMT5 vector DNA thus separated by electrophoresis (section 2.3.17) was analysed by gel imaging software gene sys. Sterile forceps were used to excise the gel fragment containing separated pMT5 vector DNA. The vector DNA in the excised gel fragment was isolated from the gel matrix using Isolate II PCR and gel commercial kit, according to the instructions thus enclosed, illustrated as follows:

- The excised gel fragment (300mg) was solubilised in 600 µl binding buffer by heating the mixture in water bath pre - set at 50◦C for 10 min, whilst hand vortexing, thus allowing the complete dissolution of the gel fragment. The gel mixture was then transferred onto silica - based gel column pre - inserted on top of a collection tube and centrifuged for 0.5 min at 11000 × *g* to bind the DNA to the column. The column containing DNA was subjected to washing using (700 µl) of wash buffer each interval. Following the washing steps, the column was centrifuged for 1 min at 11000 × *g* to dry the column.
- The column upon discarding the flow - through was transferred to a new collection tube and incorporated with DNA elution buffer (30 µl), thus provided with the kit. This was subjected to incubation in room temperature for a minute.
- Following incubation, the column was centrifuged at 11000 *× g.* The restricted DNA thus eluted was measured using nano drop and proceeded for DNA repair and ligation procedures.

2.3.19 DNA repair, purification and ligation of the pMT5 vector DNA

2.3.19.1 DNA repair

The vector DNA restriction illustrated in (section 2.3.15) resulted in generation of sticky ends in linear DNA fragment. Hence the DNA was subjected to DNA repair enzymes for forming blunt ends in the DNA, which was required for the ligation of the DNA, the ligated DNA was then subjected to sub cloning using DH5 α chemical competent cells.

Hence, the DNA purified from the agarose gel was repaired using DNA repair kit (section 2.1.19.1) as per the manufacturers protocol as illustrated as follows:

- The DNA to be repaired was mixed with 2 µl of Anza DNA repair buffer and the DNA end repair buffer (1 µl) thus provided with the Kit. This DNA - buffers mixture was then made up to 20 µl using deionised water. Following incubation at 20℃ for 15 min. The repaired DNA was then made free from impurities for further experimental studies.

2.3.19.2 DNA purification

The repaired DNA from (section 2.3.19.1) was purified from repair enzymes and other impurities with the aid of Pure link PCR purification kit according to the product protocol as illustrated as follows:

- The repaired DNA (20 µl) was mixed with 80 µl of binding buffer B2 pre - incorporated with 10 ml isopropanol.
- The reaction mixture was transferred onto pure link spin column pre - inserted onto sterile collection tube thus provided and subjected to 1min centrifugation at >10,000 × *g*. Upon discarding the flow through, the column was then rinsed with washing buffer (650 µl) and the centrifugation step was repeated for 2 min.
- Following final centrifugation, the column from previous step was inserted onto a sterile eppendrof tube. 50 µl of the elution buffer provided with the kit, was added to the centre of the column and allowed to stand for 1min and centrifuged to elute the purified DNA for ligation of the blunt ends. The purified DNA was then measured by nano drop.

2.3.19.3 Ligation of DNA blunt ends

Following purification, the DNA was subjected to ligation reactions using BIOLINE quick stick ligation kit - protocol as illustrated as follows:

- The vector DNA 66ng (12 µl) of 5.5 µg/ml concentration) from (section 2.3.19.2) was adjusted to 14 µl (4.71 µg/ml DNA concentration), as the kit protocol recommended the use of no more than 100ng DNA which was needed to be further diluted with distilled water, making up the total reaction volume to 14 µl using distilled water in a

microcentrifuge tube. 1µl of quick stick ligase and 5 µl of quick stick buffer was added to the DNA mixture upon vortexing.

- The ligation reaction was fulfilled by allowing the reaction to proceed for 15 min at room temperature.

2.3.20 Transformation of pMT5 vector DNA using DH5 α chemical competent Cells

The 5 µl of the ligated mixture was transformed into α - select competent cells using DH5 α chemical competent cells, Bioline, according to the manufacturers protocol as illustrated as follows:

- α - select competent *E-coli* cells from -80◦C storage were thawed on ice and subjected to mixing with the DNA in the ratio 1:10 µl (5 µl DNA: 50 µl competent *E-coli* cells). The same was conducted with reference DNA pUC 19 thus provided with the kit.
- Following 30 min incubation in ice, the DNA - competent cell mixtures were subsequently incubated in water bath pre - set at 42℃ for 30 s. The DNA - competent cell mixture of the pMT5 vector and the pUC 19 control DNA was then allowed to cool in ice for 2 min.
- The DNA - competent cell mixtures were subsequently made up to 1 ml using 900 µl of sub cloning medium SOC. Following dilution, the cultures were incubated in a shaking incubator pre - set at 37℃ at 200 rpm for 1 h.
- Following incubation, 100 µl of the transformation mixtures were spread plated on Petri dishes containing Luria-Bertani agar prepared with ampicillin antibiotic (1% w/v).
- The inoculated plates were incubated for 24 h at 37℃.

The pUC 19 control plasmid provided with the α - select chemically competent strains were used as positive refence. The pUC 19 transformation reaction mixture (5 µl) was inoculated onto sterile petri plates containg SOC sub-cloning medium pre - supplemented with ampicillin antibiotic by spreading 5 µl of the inoculum.

2.3.21 Transfection of the p21 plasmid DNA and vector DNA onto HT-29 cancer cell lines

The cancer cell lines HT-29 grown in 75 cm^2 tissue culture flasks were trypsinated using trypsin EDTA as illustrated in (section 2.3.1.2). The cell count was determined using haemocytometer (section 2.3.3). Cells at cell density of 1×10^6 cells were seeded onto each well of the sterile 6 well assay plates, HT-29 cell lines were allowed for attachment for 24 h in incubator at 37◦C. Following incubation, the cells were resuspended with EMEM culture medium devoid of FBS.

Batch cultures of Luria-Bertani broth containing p21 plasmid DNA or vector DNA (thus obtained by transformation reaction) were prepared for plasmid DNA isolation. The Plasmid DNA were quantified using nanodrop. The vector DNA was measured to be 80.2 µg/ml and p21 plasmid DNA was (118.3 µg/ml) in concentration. EMEM culture medium devoid of FBS was used for the transfection as the FBS may interfere with the transfection using lipofectamine 2000. The transfection using lipofectamine 2000 was performed as per the manufactures protocol, illustrated as follows:

4.0µg of plasmid DNA and the vector DNA was subjected to dilution in 250 µl EMEM medium separately. Lipofectamine 10 µl was also simultaneously diluted in EMEM medium. Following a 5 min incubation, both the DNA in EMEM medium and lipofectamine in EMEM medium were mixed together. The DNA - lipofectamine mixture could form complexes by incubating at room temperature for 20 min. The DNA - lipofectamine complexes (500 µl) were then added to HT-29 cells, which had been pre-seeded in 6 well assay plates containing 1.5 ml of EMEM medium.

2.3.22 Statistical analysis

Statistical analysis was performed using graph - pad (version7) software. Data were analyzed statistically using one - way ANOVA with post hoc Sidaks multiple comparisons test, Turkey's multiple comparisons test and paired T- test accordingly.

Chapter 3

Effect of antimycotic/antibiotic on colorectal cancer cell line HT-29

3.1 Introduction

The antimycotic/antibiotic solution at recommended dosage are incorporated in cell culture medium to avoid mammalian cell lines from microbial or fungal contaminations in *in vitro* experimental studies, however these agents at high dosage are reported to disrupt the cells semi - permeable membrane layers, hindering the normal metabolic activities of cancer cells including mRNA translation processes finally inducing cytotoxicity (Kuhlmann. 1995; Llobet et al. 2015).

In case of *in vitro* gene transfection studies, using commercial transfection reagents such as lipofectamine 2000, the antibiotics incorporated in cell culture medium may tend to form complexes with the transfection reagent. These agents if transported *in vitro* may induce toxicity, hampering successful gene expressions. Hence considering, the undesirable effects of these agents, their use are highly unsuitable for *in vitro* experimental transfection studies (www.thermofisher.com).

Report evidence the anti-cancer activity of broad category of commercial antibiotics in obstructing the mitochondrial pathway in cancer tissues and arresting further proliferation of several cancer types. Antibiotic such as Doxycycline in combination with natural vitamins such as ascorbic acid evidence to target the mitochondria of cancer cells, which adversely affect the translational processes, resulting in cellular instability. These anti-cancer agents not only arrest cancer growth but also attenuate cancer stem cells, preventing future reoccurrences according to recent investigation (De Francesco et al. 2017). Moreover, cancer cells are also reported to experience cellular stress upon exposure to antibiotic such as thiostrepton, which elevated the expression of HSPA1A (Sandu et al. 2014).

Although the antibiotics serve as effective anti-cancer agents, the after effects generated by these compounds upon prolonged administration are of high consideration. Studies confirm not only the deleterious effects of these compounds in damaging human intestinal walls, but also the elimination of the intestinal microbial flora that plays a key role in the digestion

processes in the human intestinal system (Modi et al. 2014). More over population - based research outlines the probability of developing bowel malignancies in patients subjected to antibiotic exposure for prolonged periods (Kilkkinen et al. 2008) thus suggesting the negative impact of using antibiotics in conditions like cancer which requires prolonged administration of these agents**.**

3.1.2 Aims and hypotheses

This chapter includes the following aims and hypotheses:

1. To elucidate the effect of antimycotic/antibiotic on colorectal cancer cell line HT-29.

Hypotheses-

H0 antimycotic/ antibiotic do not inhibit the growth of HT-29 cells.

H1 H0 antimycotic/ antibiotic may inhibit the growth of HT-29 cells.

2.To analyse the interaction of Heat shock protein HSPA1A in response to antimycotic/ antibiotic exposure on HT-29 cell lines.

Hypotheses-

H0 antimycotic/antibiotic will not produce any intracellular or extracellular stress on the growth of HT-29 cells.

H1 antimycotic/antibiotic may induce intracellular or extracellular stress on the growth of HT-29 cells.

3.2 Methods

3.2.1 Cell seeding and experimental design

Adherent colorectal cancer cell lines HT-29 at seeding density 5×10^5 cells/ml were used in all experiments (section 2.3.1.2).

3.2.2 Treatment with antimycotic/antibiotic

Cells were treated with varying concentrations of antimycotic/antibiotics. Untreated cells were placed as controls.

3.2.3 Measurements of cell viability

The cells were analyzed for cytotoxicity by MTS (Section 2.3.4) and PI assays (Section 2.3.5) following 24 h incubation. Flow cytometric analysis were also used to confirm the percentage of apoptosis and necrosis by annexin V and PI staining method (section 2.3.6).

3.2.4 Determination of HSPA1A protein expression

The colorectal cancer cell line HT-29 upon treatment with varying concentration of anti-mycotic/antibiotics for 24 h were evaluated for HSPA1A protein expression at both intracellular and cell surface levels by flow cytometry (section 2.3.7.1, 2.3.7.2).

3.3 Results

3.3.1 Determination of apoptosis and necrosis

The following experiments were performed to evaluate the effect of antimycotic/antibiotic on the growth of colorectal cancer cell lines HT-29. The MTS cell viability assay presented a gradual decrease in cell viability upon treatment with antimitotic/antibiotics in a concentration dependent manner ($P<0.0001$, Fig 3.3.1A, Table 3.1). The results from the PI assay showed no significant increase in PI uptake, suggesting the effect may be due to apoptosis (Fig 3.3.1B, Table 3.1) rather than necrosis.

The cells treated with the antibiotics were confirmed for apoptotic cell death using annexin V and PI staining assay by flow cytometry which confirmed the increased rate of apoptosis with statistical significance as high as 89.7% (Fig 3.3.2,3.3.3) of late apoptosis and 2.8% early apoptosis of HT-29 cells.

3.3.2 Evaluation of HSPA1A expression profiles post antimycotic/ antibiotic on HT-29 cancer cell lines

The HSPA1A stress proteins were studied to be released upon antimycotic/antibiotic treatment. The study analyzed the expression of HSPA1A at both intracellular as well as cell surface levels. The intracellular HSPA1A results (Fig 3.3.4, 3.3.5) presented no significant variation on the HSPA1A status compared to the untreated controls.

On the other hand, the expression of cell surface HSPA1A protein were measured (Fig 3.3.6, 3.3.7) to be significantly higher with that of the control cell line and the expression increased with increase in concentration of the antimycotic/ antibiotic in HT-29 colorectal cell lines.

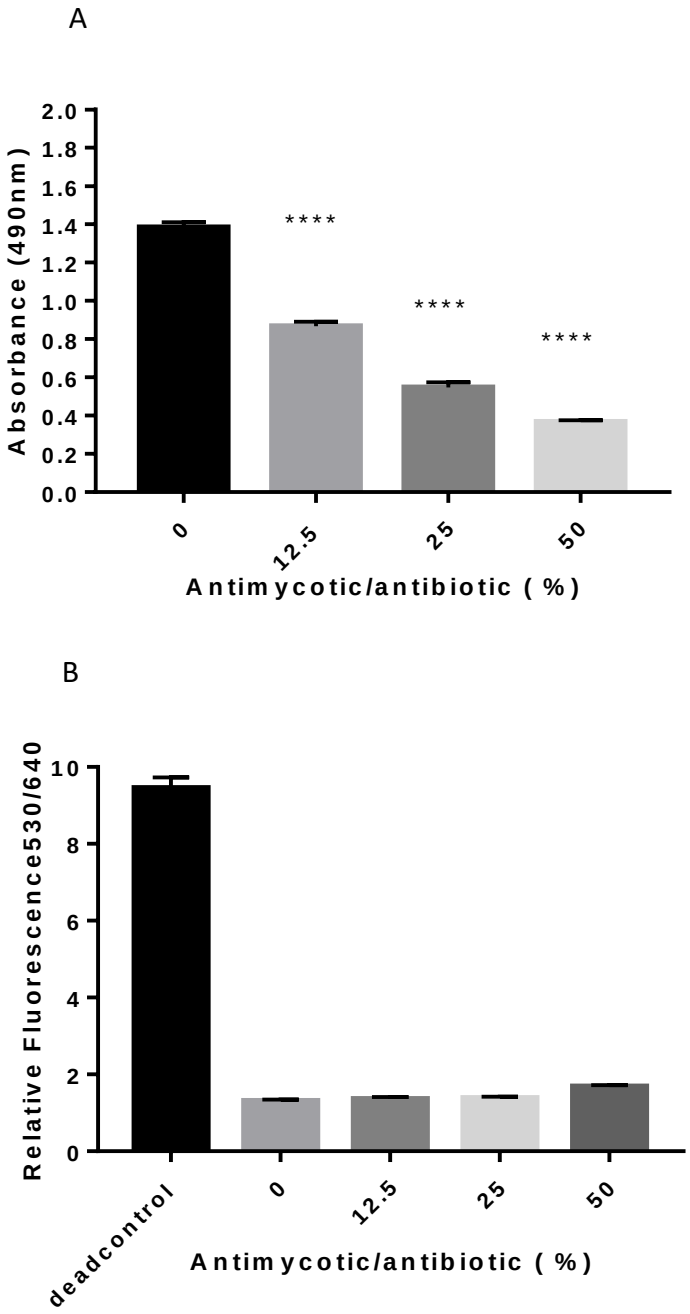


Fig: 3.3.1 Effect on cell viability by antimycotic/antibiotic on HT-29 cell lines. Cell lines were seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) and treated with varying concentration of antimycotic/antibiotic. The cells were incubated for 24 h in incubator at 37°C, cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one- way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences of untreated and different anti - mycotic /antibiotic concentrations are indicated by **** ($p<0.05$, mean±SD; n=4).

Table 3.1 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
Untreated vs Anti - mycotic /antibiotic	**Significance**	**P value**	**Untreated vs Anti - mycotic /antibiotic/Dead**	**Significance**	**P value**
0 vs 12.5	****	P<0.0001	0 vs dead control	****	P<0.0001
0 vs 25	****	P<0.0001	0 vs 12.5	ns	P 0.9803
0 vs 50	****	P<0.0001	0 vs 25	ns	P 0.9251
			0 vs 50	ns	P 0.9990

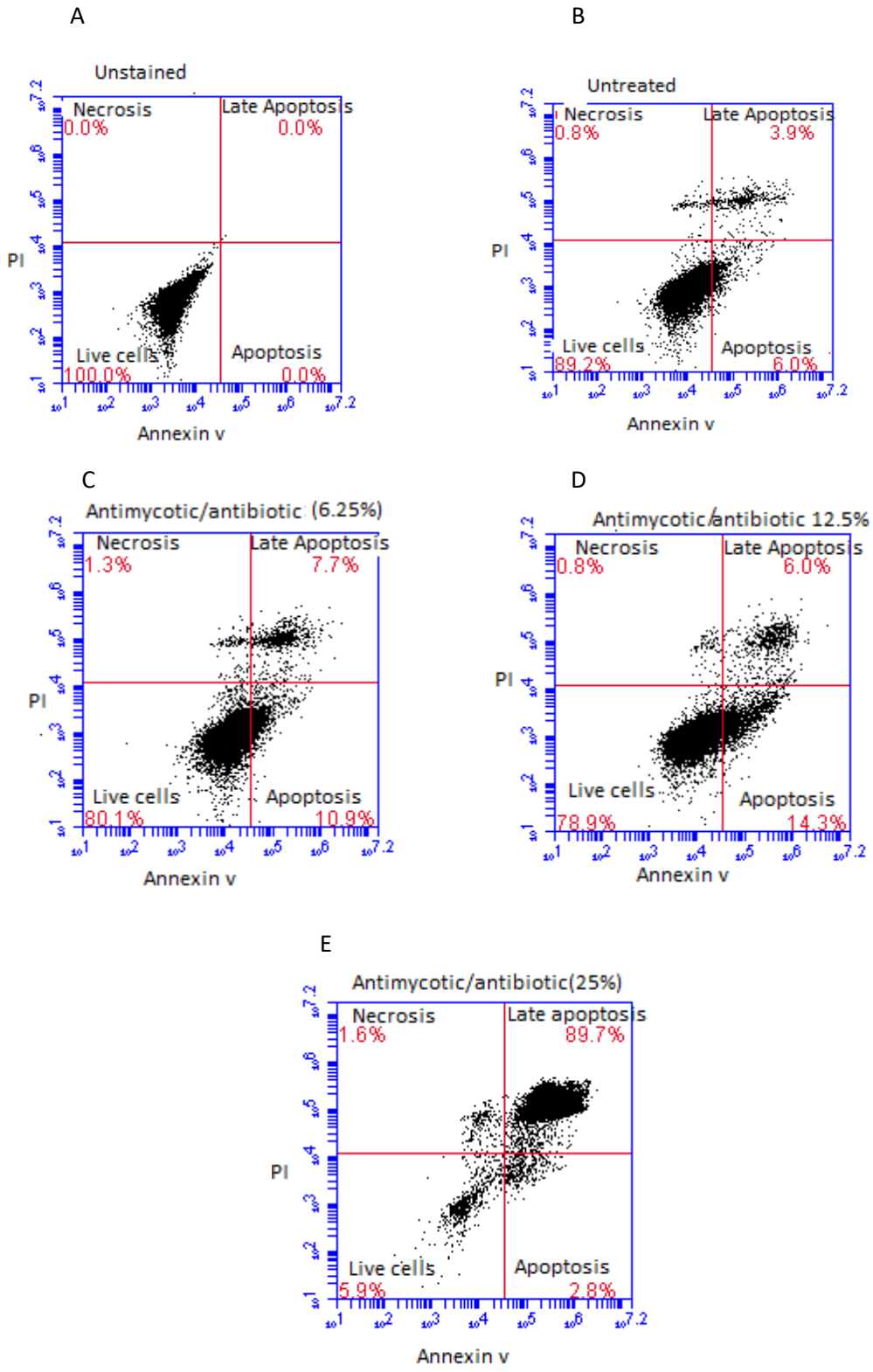


Fig: 3.3.2 Flowcytometric data presentation of annexin V and PI staining in HT-29 cell lines exposed to antimycotic/ antibiotic. HT-29 cell lines at cell density (1 × 10^6 cells), the cells were subjected to antimycotic/ antibiotic treatment and were allowed for incubation at 37◦C for 24h. The cells were then analyzed for apoptosis by annexin V and PI staining using flow cytometer- (A) unstained control, (B) untreated (C) 6.25%, (D) 12.5%, (E) 25%.

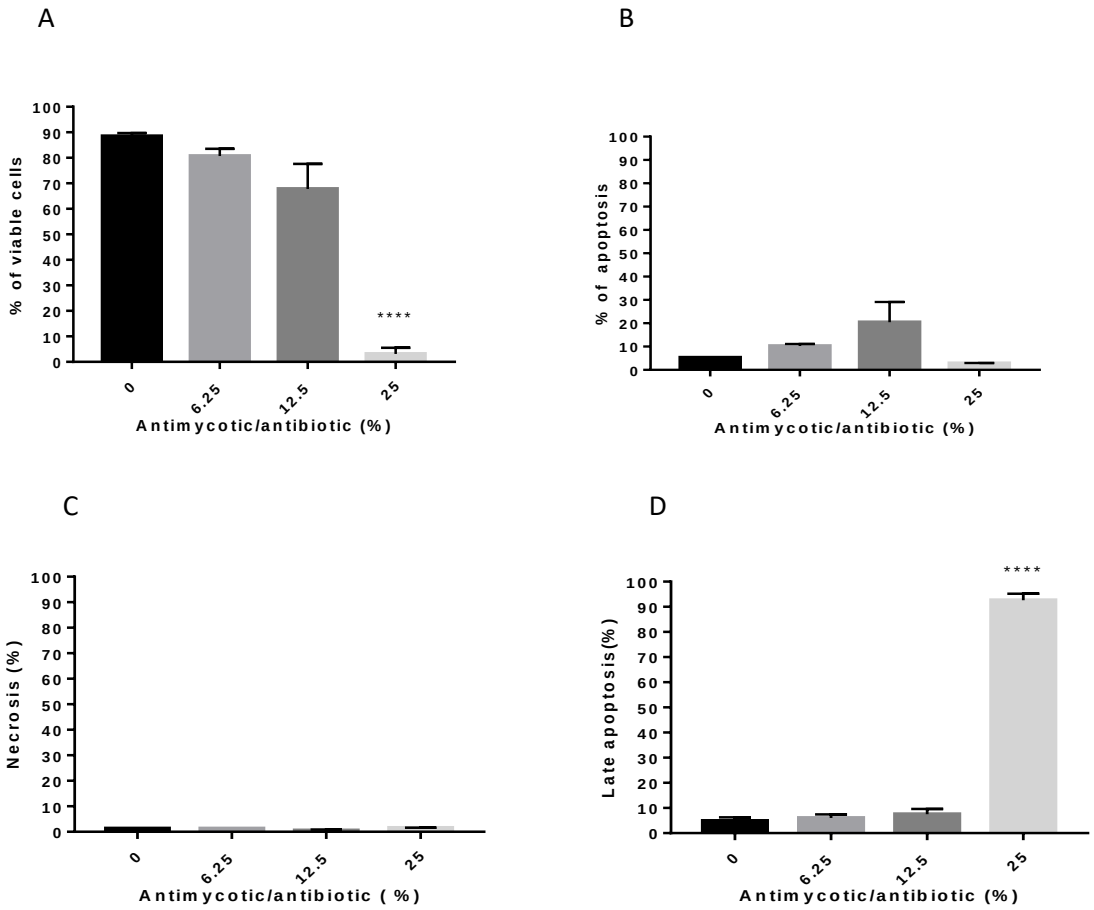


Fig: 3.3.3 Effect on cell viability by antimycotic /antibiotic on HT-29 cell lines by annexin V and PI staining. Cell lines were seeded at cell density of (1×10^6 cells) onto 6 well assay plates and treated with varying concentration of anti - mycotic/antibiotic. The cells were incubated for 24 h in incubator at 37°C. Cell viability was measured by annexin v and PI staining by flow cytometer. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences of untreated and different concentrations are indicated by **** ($p<0.05$, mean±SD; n=3).

Table 3.1 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

Annexin v and PI staining assay (Control vs antimycotic antibiotic concentration) (ns* insignificant)

% of viable cells	Significance	P value	% of early apoptosis	Significance	P value
0 vs 6.25	ns	P 0.0840	0 vs 6.25	ns	P 0.1303
0 vs 12.5	ns	P 0.2129	0 vs 12.5	ns	P 0.4112
0 vs 25	***	P 0.0007	0 vs 25	ns	P 0.223
% of necrosis	**Significance**	**P value**	**% of late apoptosis**	**Significance**	**P value**
0 vs 6.25	ns	P 0.9978	0 vs 6.25	ns	P 0.9077
0 vs 12.5	ns	P 0.297	0 vs 12.5	ns	P 0.8553
0 vs 25	ns	P 0.380	0 vs 25	ns	P< 0.0001

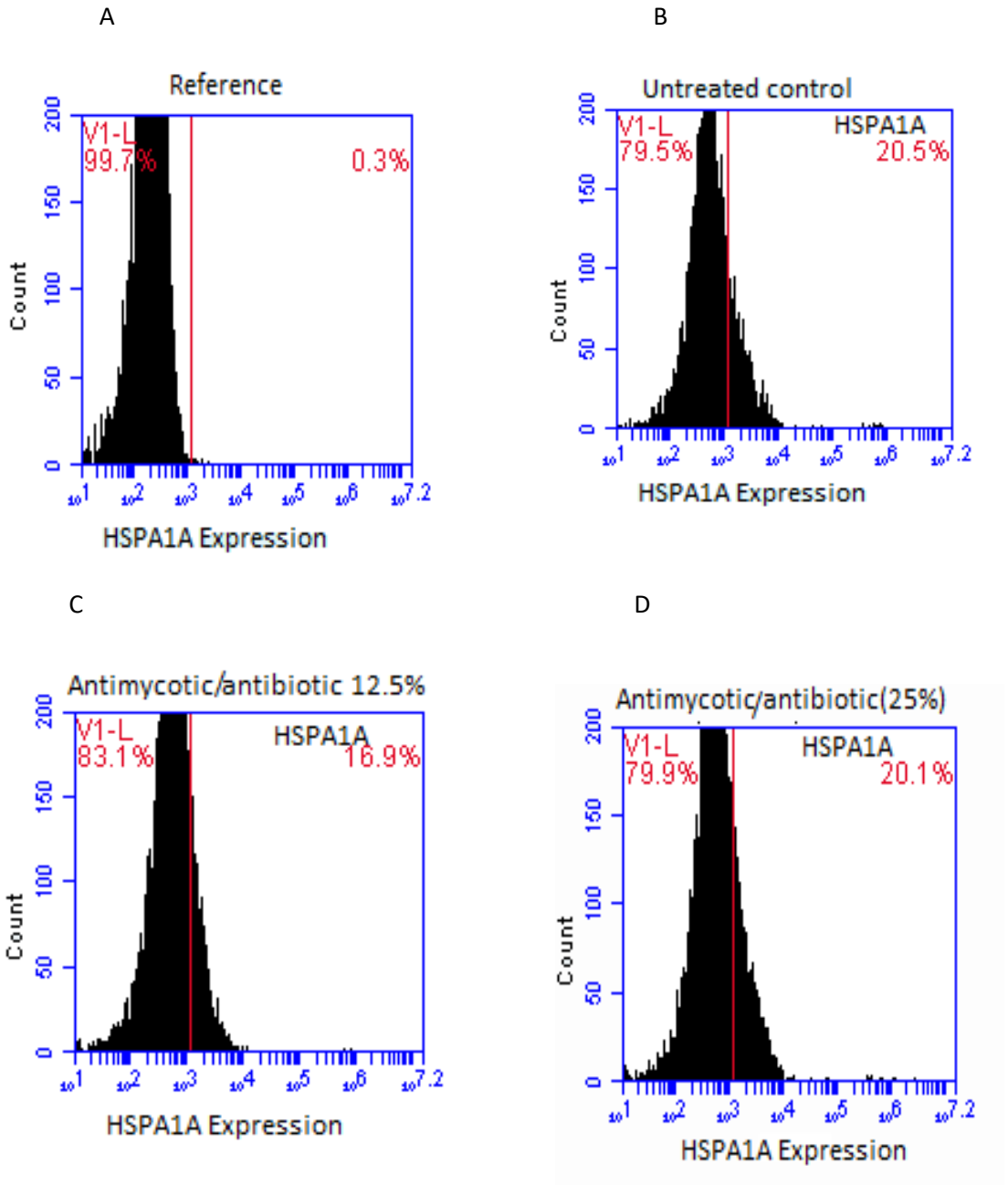


Fig: 3.3.4 Intracellular HSPA1A expression resulted from antimycotic/ antibiotic treatment in HT-29 cell lines.

HT 29 cell lines were seeded at cell density of (1×10^6 cells) onto 6 well assay plates and treated with varying concentration of anti – mycotic/antibiotic. The intracellular HSPA1A expression was analyzed using flow cytometer. Flow cytometry data presentation of histograms gated with primary antibody stained control(A)(B); untreated controls(C); 12.5% concentration (D); 25% concentration.

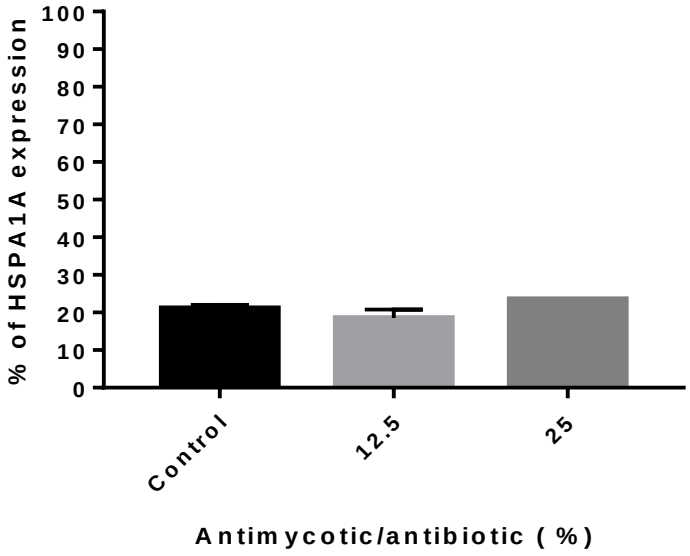


Fig: 3.3.5 Graphical representation of Intracellular HSPA1A expression resulted from antimycotic/antibiotic treatment in HT-29 cell line. Statistical analysis performed using one - way Anova represented mean±SD(P<0.05) n=3, depicted insignificant variation in HSPA1A expression between the control and treated groups expressing p values; control vs 12.5(p0.2890); 12.5 vs 25% (p0.0749).

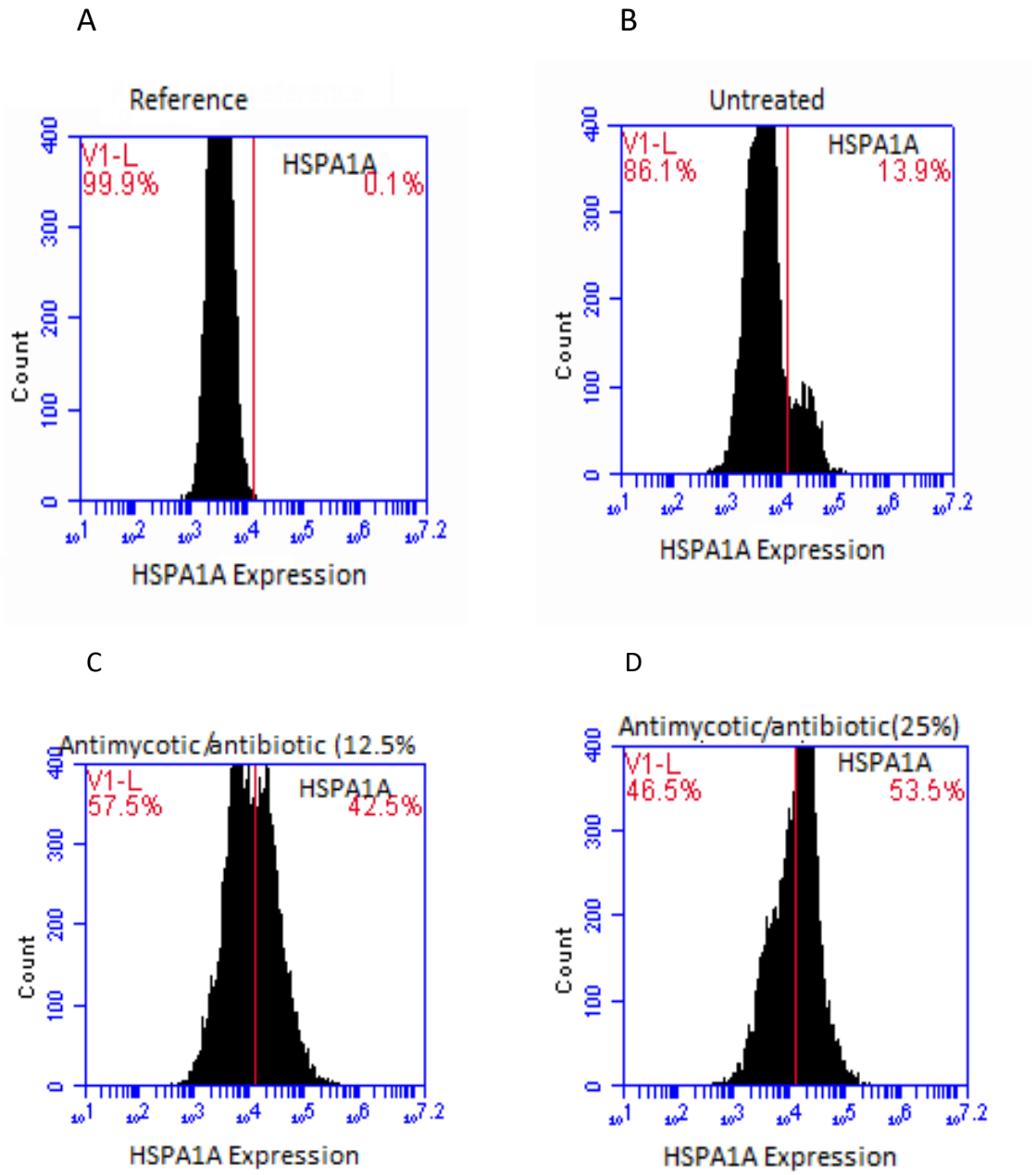


Fig: 3.3.6 Cell surface HSPA1A expression resulted from antimycotic/ antibiotic treatment in HT-29 cell lines

HT 29 cell lines were seeded at cell density of (1×10^6 cells) onto 6 well assay plates and treated with varying concentration of antimycotic/antibiotic. The cell surface HSPA1A expression was analyzed using flow cytometer. Flow cytometry data presentation of histograms gated with primary antibody stained control (A); untreated controls (B); 12.5% concentration (C); 25% concentration (D).

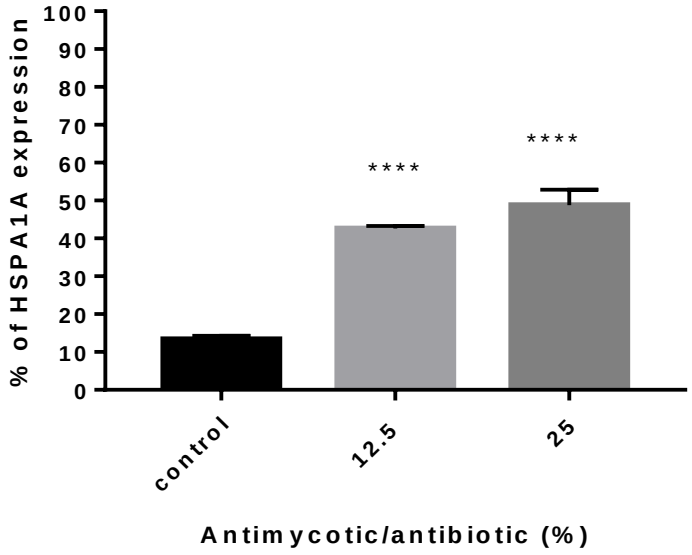


Fig: 3.3.7 Graphical representation of surface HSPA1A expression resulted from antimycotic/antibiotic treatment in HT-29 cell line. Statistical analysis performed using one - way Anova represented mean±SD(P<0.05) n=3, depicted no significant difference in HSPA1A expression between the control and treated groups expressing p values; control vs 12.5 (p<0.0001****); control vs 25 (p<0.0001****).

3.4 Discussion

The aim of this chapter was to evaluate the effect of antimycotic/ antibiotic on colorectal cancer cell lines HT-29. Previous studies have evidenced the negative impacts of using anti-mycotic/antibiotics in mammalian tissues (Kuhlmann 1995; Llobet et al. 2015), which has shown to interfere with *in vitro* experimental outcomes. In case of *in vitro* experiments using nanoparticles or microparticles, the antimycotic/ antibiotic used in mammalian tissue culture medium, may get encapsulated within these delivery systems and reach the cellular compartments at undesirable amounts, affecting experimental results.

The penicillin and streptomycin combination are also reported to induce genes, mainly ATF3-Activating transcription factor 3, which are studied not only to deregulate gene transcription but also involved in dephosphorylation of proteins, affecting protein synthesis in HepG2 liver cell lines *in vitro* (Ryu et al. 2017). The proliferation of cultured mammalian cell lines is also studied to be adversely affected by antibiotics, showcasing enhanced proliferation of cultured cell lines in antibiotic free culture medium compared to that grown in antibiotic enriched medium (Ricarda et al 2013).

Besides, the adverse effect of antibiotics in mammalian cell culture, research report have previously stated the extensive application of several anti - microbial agents in arresting several cancer types (De Francesco et al. 2017). The results from this study also reports the apoptotic activity of these compounds in a dose dependent manner in HT-29 cell lines (Fig 3.3.1,3.3.2).

Penicillin - streptomycin antibiotics play an active role in altering the expression profiles of several genes which are exclusively linked with the transcription process and stress responses in HepG2 liver cell lines (Ryu et al. 2017). The results in this chapter were shown to trigger an increase in external HSPA1A protein expression in a dose dependent manner (Fig 3.3.6, 3.3.7), which may act as alarm signals for attracting host immune cell response, finally triggering cancer cell death. The HSPA1A expression profiles in this study matches other studies which presented a similar response of HSPA1A as in case of hyperthermia treatments and HSPA1A stress response induced by antibiotic thiostrepton (Jolesch et al. 2012; Sandu et al. 2014). However, antimycotic/ antibiotics did not induce the expression of HSPA1A proteins at the

intracellular regions, suggesting the treatment reflected no stress induction internally (Fig 3.3.4, 3.3.5).

Although antibiotics evidence to arrest several cancer malignancies, several reports at same time express concerns regarding the side effects of these agents, which contribute to the risk of acquiring bowel cancers due to prolonged intake of high dose antibiotics (Cao et al. 2017). Moreover, population based studies show that more than 5 course administration of several class of antibiotics such as penicillin's and tetracyclines, showed approximately 0.9% of the tested population at risk of acquiring prostate and breast malignancies (Boursi et al. 2015; Friedman et al. 2006).

The antibiotics intake adversely affect the survival of intestinal microflora by hampering the synthesis of peptidoglycan - an inevitable polymer required for the formation of bacterial cell wall (Mucsi et al. 2013). Reduced microbial flora in turn resulted in increasing the pH of the intestinal region, which is one of the factors favoring colorectal cancer initiation (Ohigashi et al. 2013). Antibiotic treatments are shown to hamper the metabolic processes and protein synthesis in gut microbes, in turn disrupting their growth. Previous studies also highlight the adverse effect of antibiotic persistence, which prevented the survival of intestinal microflora for prolonged duration of several years for a normal recovery (Mai et al. 2013). This late recovery of the gut microflora in antibiotic treated patients were shown to enhance the survival of microbial pathogens such as vancomycin resistant enterococcus species, which resulted in infection and sepsis (Ubeda et al. 2010).

The experimental outcomes from previous studies having demonstrated the adverse effects of antibiotics and the results presented in this chapter, collectively not only addressed the growth inhibitory effect of antimycotic/ antibiotic in HT-29 cell lines (Fig 3.3.1,3.3.2), but also showed the interference of these agents in inducing external stress response in culture cell lines (Fig 3.3.6, 3.3.7), which may in turn affect *in vitro* experimental results. Therefore, all the experiments in this thesis were performed with cancer cell lines maintained in culture medium devoid of antimycotic/ antibiotics as this may interfere with the microgel or transfection studies performed throughout the thesis. Hence, considering the negative impacts generated by the presence of antibiotics in tissue culture medium, it`s use is not recommended for mammalian cell culture (Ricarda et al. 2013; Ryu et al. 2017).

Chapter 4

Inhibition of HSPA1A in combined therapies

4.1 Introduction

Radiation exposure is widely accepted as first line conventional treatment strategy for detaining cancer progression. According to studies at the molecular level, these ionizing radiations disrupt the genetic material (Maier et al. 2016), leading to the activation of tumour suppressor genes directing cells towards apoptosis. Excessive oxidative stress creates a cellular imbalance in cells exposed to gamma radiation, finally resulting in cell death (Lee et al. 2017). Although radiation therapy effectively destroys cancer cells, the resistance to some cancer cells (De Bacco et al. 2011) and the undesirable side effects produced due to prolonged exposure of radiation is of high consideration, hence to overcome the side effects, researches are aiming at improving the therapeutic index of this therapy by limiting duration of radiation exposure and applying it in combination with chemotherapeutics. One factor which may result in resistance to apoptosis is the elevated expression of HSPA1A proteins in cancer cells (Sherman et al. 2015). The transcriptional activation of HSPA1A is studied to be dependent on the acetylated status of this protein, HSPA1A is studied to be acetylated by ARD1 acetyl transferase which in turn determines its function in maintaining the conformational status of proteins, on the other hand HSPA1A deacetylation works in ubiquitination of proteins within mammalian cells (Seo et al. 2016). Moreover, HSPA1A generated from stress response act as alarm system, eliciting the natural defense mechanism, or in some instances, their expression works to demote natural immunity, allowing the progression of malignant cells. Cellular contents released as result of necrosis, allows the intracellular heat shock proteins reach the surface of cell membrane, which signals the immune system, identify the threat and act accordingly (Klink et al. 2012).

Studies have explored the effect of HSPA1A protein inhibitor compounds Phenylethylsulphomide (Pifithrin - μ) and 2-(3-chlorophenyl) ethynesulfonamide (Pifithrin chloride), hindering autophagy - which is a lysosome enzyme catalysed mechanism and plays a major role in discarding of unwanted metabolic residues within cancer cells thus allowing successful proliferation (Mizushima et al. 2008; Jones et al. 2016). Previous report with

pifithrin - α and pifithrin - β reported to express a protective role, against hampering UV induced apoptosis in embryonic testicular carcinoma (Keller et al. 1999), while other researcher presented accelerated apoptotic effects with pifithrin - α in combination with radiation treatment in ovarian and colorectal cancers (Walton et al. 2005). However, reports of pifithrin - μ in combination with UV has not been reported yet. Hence the first phase of this chapter concentrated on addressing the combined effect of UV radiation with pifithrin - μ in the leukaemia cell line U937 and the colorectal cancer cell line HT-29.

Interestingly, given the importance of HSPA1A proteins in cancer cells, hyperthermia is another treatment modality, which evidence cytotoxicity at elevated temperature as high as 42℃ in in several cancer types (Jolesch et al. 2012). Temperature fluctuation in the cancer environment due to hyperthermia results in increased release of HSPA1A proteins, which assist in the activation of immune cells towards the destruction of cancer cells releasing HSPA1A (Horowitz. 2007).

Introducing heat with the aid of electronic devices has been employed for the treatment of solid malignancies. Electro hyperthermia has been extensively tested as a cancer therapeutic strategy, studies demonstrate increased survival rates in relapsed tumour conditions upon administration of electro hyperthermia in combination with radiotherapy and chemotherapeutics such as sorafenib in hepatic malignancies (Gadaleta et al. 2014).

Report demonstrate hyperthermia induced cytotoxicity at elevated temperatures ranging from 40 - 44℃ in solid malignancies (Van der Zee. 2002), hyperthermia administration for an hour duration at 41.5℃ alongside combination therapies also shown to enhance the cancer cells capability of increased expression rate of HSPA1A proteins, making chemotherapy and radiation therapies more effective *in invitro* colorectal cancers in human and mouse models (Schildkopf et al. 2011).

The experiments in this chapter will investigate the treatment effects of UV radiation, pifithrin - μ and pifithrin chloride or hyperthermia at 42℃ , which may induce cytotoxicity in both leukaemia cell line U937 and the colorectal cancer cell line HT-29. This study also evaluated whether pifithrin - μ at low dosage may accelerate the effect of UV radiation in both the cancer cell lines, which may reduce the risk of prolonged radiation exposure, enhancing the therapeutic potential of radiation therapy for cancer treatments.

4.1.1 Aims and hypotheses

This chapter evaluated the potential for using inhibitors of HSPA1A protein activity in combination with UV or temperature.

The objectives of the study are:

1. To evaluate the cytotoxicity induced by UV radiation, pifithrin - μ and pifithrin chloride in the leukaemia cell line U937 and the colorectal cancer cell line HT-29.

Hypotheses-

H0 UV radiation, pifithrin - μ and pifithrin chloride treatment will not induce cell death in leukaemia cell lines U937 and colorectal cancer cell lines HT-29.

H1 HSPA1A inhibitors - pifithrin chloride and pifithrin - μ and UV treatments will arrest the growth of U937 and HT-29 cells.

2. Investigate whether cytotoxicity could be enhanced by applying UV radiation with pifithrin - μ at low dosage in both the cancer cell lines used in the study.

Hypotheses-

H0 the HSPA1A protein inhibitor pifithrin - μ in combination with UV will not enhance the effect of UV radiation in arresting cell growth at limited time of UV exposure in both the cancer cell lines.

H1 the HSPA1A protein inhibitor pifithrin - μ in combination with UV may enhance the effect of UV radiation in arresting cell growth at limited time of UV exposure in both the cancer cell lines.

3. Study the effects of hyperthermia cytotoxicity in the leukaemia cell line U937 and the colorectal cancer cell line HT-29.

Hypotheses

H0 hyperthermia treatment at 42℃ will not arrest the growth or induce stress response in U937 and HT-29 cell lines.

H1 hyperthermia at 42℃ will induce stress response and arrest the growth of both U937 and HT-29 cells.

4.2 Methods

4.2.1 Cell seeding and experimental design

Cells were cultured as described in (section 2.3.1.2). Cells at seeding density 5×10^5 cells/ml were used in all experiments.

4.2.2 UV irradiation exposure

The cells pre - seeded onto 96 well plated were carefully exposed to UV radiation at low voltage by moving the plates across the UV light, in a time dependent manner. The cells following treatment were incubated for 24 h duration at 37℃. Unexposed cells were placed as controls.

4.2.3 Hyperthermia

Aliquots of 3 ml of cells in suspension were introduced into 10 ml sterile tubes and subjected to 42℃ for 1 h. The cells were then allowed to recover at 37℃ for 2 h. The cells were immediately plated onto 96 well assay plates and incubated for 24 h duration at 37℃. Cells subjected to treatment at 37℃ were placed as controls.

4.2.4 Treatment with pifithrin - μ and pifithrin chloride

Cells were treated with pifithrin - μ and pifithrin chloride at varying concentrations prior to UV or temperature treatment. The cells following treatment were incubated for 24 h or 48 h duration at 37℃ accordingly. Unexposed cells were placed as controls. Dead cells were placed as dead controls

4.2.5 Measurements of cell viability

The cells were analyzed for cytotoxicity by MTS and PI assays (Section 2.3.4, 2.3.5) following 24 h and 48 h incubation duration. The effect of UV along with pifithrin - μ treatments were also observed in both the cancer cell lines.

Flow cytometric analysis were also used to confirm the percentage of apoptosis and necrosis by annexin V and PI staining method (Section 2.3.6).

4.2.6 Determination of hyperthermia induced HSPA1A proteins

The cancer cell lines U937 and HT-29 post hyper thermic treatments were evaluated for HSPA1A protein expression at cell surface levels by flowcytometry (section 2.3.7.2).

4.3: Results

4.3.1: Effect of UV on U937 cell lines

The following experiments were conducted to determine the effect of UV radiation exposure (0, 2, 4, 6 and 8 s) on U937 leukaemia cell line. Following UV treatment cells were incubated for 24 h at 37℃. The MTS assay results displayed that increased UV exposure resulted in a decline in cell viability in cells exposed for 4 s or longer (Fig 4.3.2A, Table 4.1). Microscopic observation also displayed cytotoxicity with increase in exposure time (Fig 4.3.1). However, at no time was necrosis observed by PI (Fig 4.3.2B).

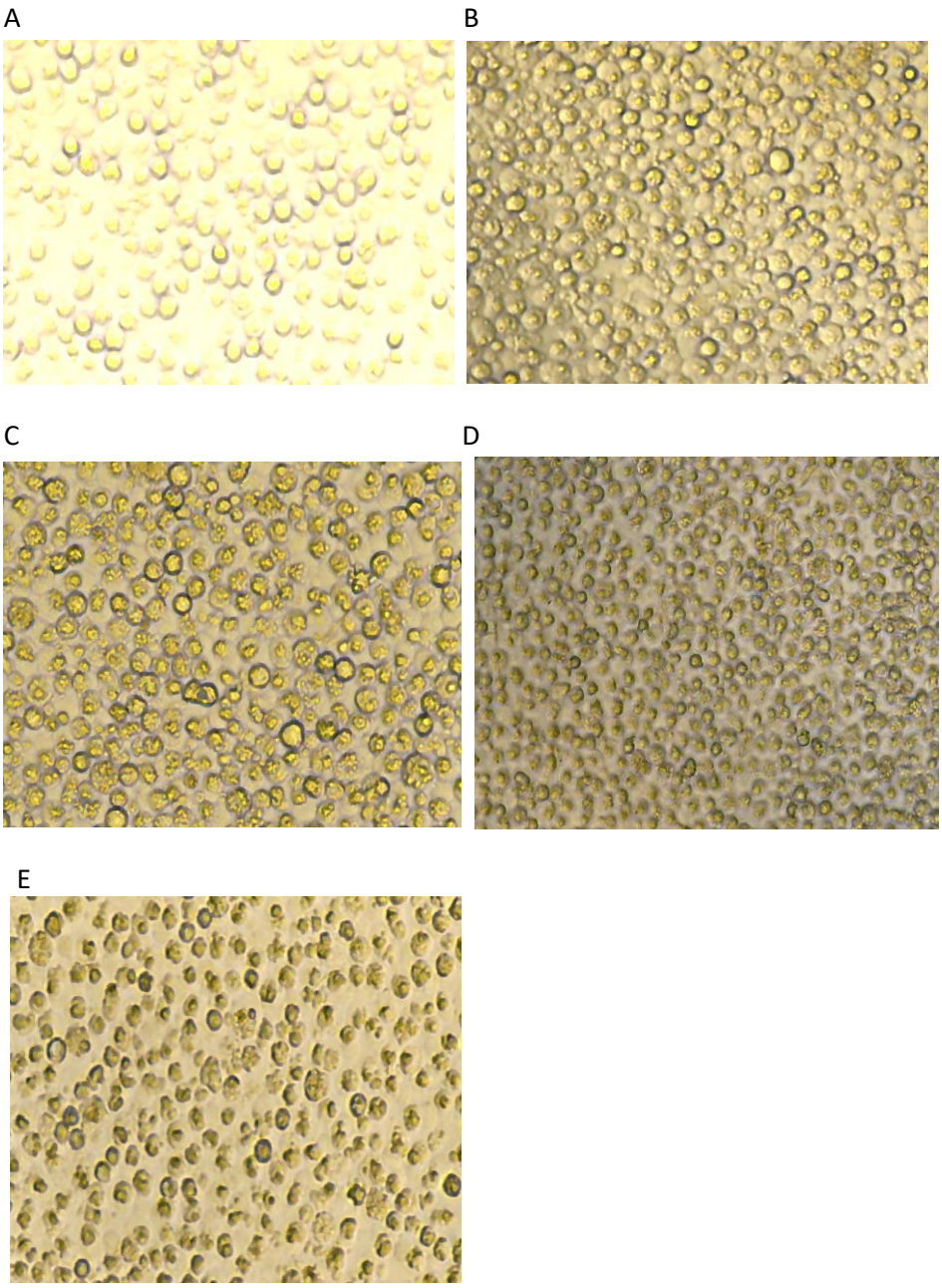


Fig: 4.3.1 Microscopic observation of U937 cell lines treated with UV radiation at magnification of 20X objective (2048 × 1536 px). The cells following treatment followed by incubation for 24 h at 37℃ were microscopically observed for cytotoxicity; (A) untreated, (B) 2s, (C) 4s, (D) 6 s and (E) 8 s of UV exposed.

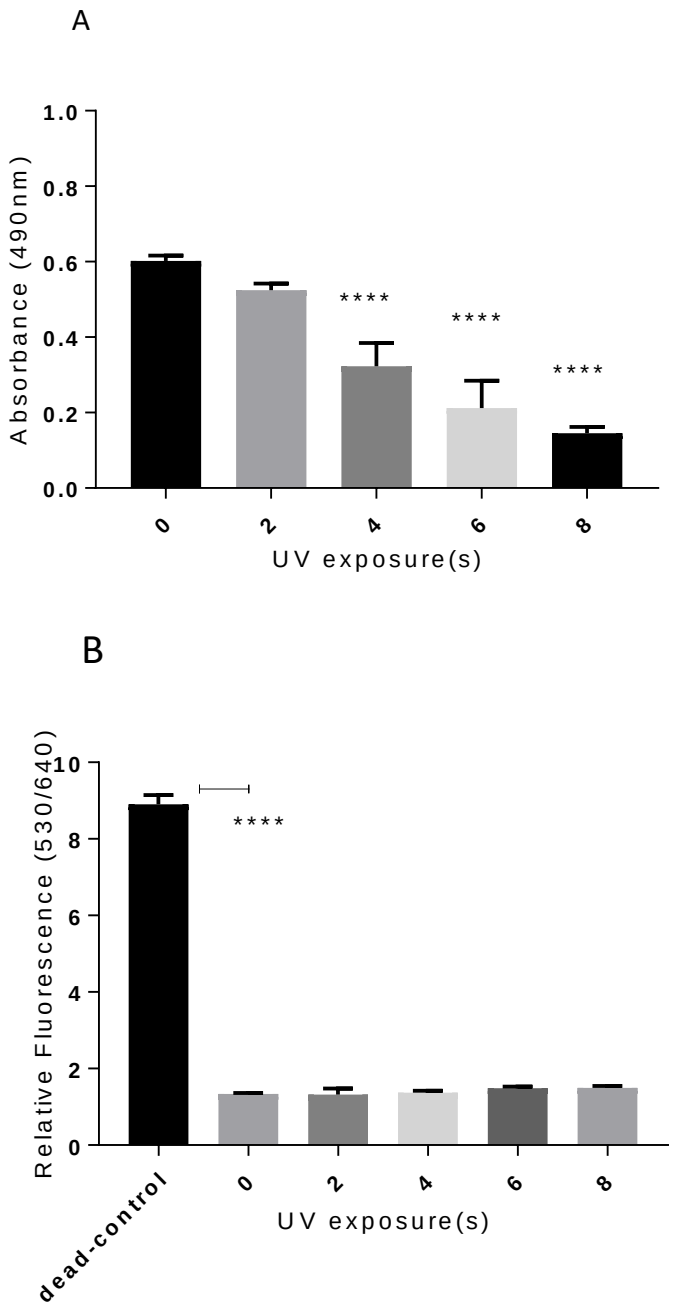


Fig: 4.3.2: The effect on cell viability by UV exposure on U937 cell lines. U937 cell lines seeded onto 96 well assay plates at cell density (5×10^5 **cells/ml**) were exposed to UV light at varying time intervals of 2,4,6,8 s. The cells were allowed for 24 h incubation at 37°C, cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences from the 0 UV exposure are indicated by **** ($p<0.05$, mean±SD; n=4).

Table 4.1 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

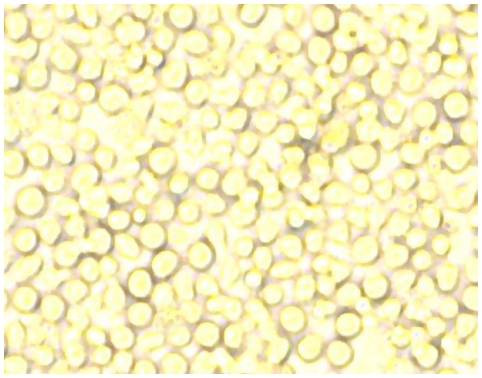
MTS Assay			**PI Assay**		
UVexposure time (s)	**Significance**	**P value**	**UVexposure time (s)**	**Significance**	**P value**
0 vs 2	ns	P 0.1515	0 vs 2	ns	P>0.9999
0 vs 4	****	P<0.0001	0 vs 4	ns	P 0.9971
0 vs 6	****	P<0.0001	0 vs 6	ns	P 0.5109
0 vs 8	****	P<0.0001	0 vs 8	ns	P 0.4793

4.3.2 Effect of pifithrin - μ and pifithrin chloride on U937 cell lines

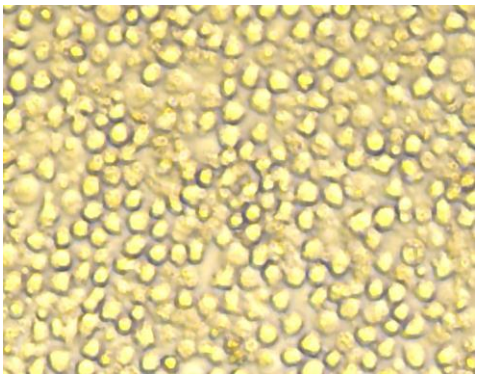
U937 cell lines were subjected to treatment with pifithrin - μ at varying concentrations 25,50 and 100μM and allowed for incubation for 24 h and 48 h. The cells were then analyzed by MTS and PI assays. The cells displayed a progressive decline in cell viability at all the treated concentrations within 24 h (Fig 4.3.4 A) and by microscopic examination (Fig 4.3.3) in contrast to the untreated controls. The data showed no significant difference between treated concentrations 25 and 50 μM (P 0.6374, Table 4.2), while the 50 μM expressed significant variation with the next higher concentration 100 μM (P 0.0040**, Table 4.2). PI assay (Fig 4.3.4 B) showed necrotic effect with increase in pifithrin - μ concentrations exhibiting significance of (P<0.0001) Table 4.2) at 100μM with respect to next lower concentration 50 μM of pifithrin - μ used in the study. Upon prolonged exposure of the pifithrin treatment for 48 h, the MTS assay expressed no alteration from the effect presented by 24 h treatment (Fig 4.3.5 A, Table 4.3) while the PI assay displayed reduced necrosis after 48 h pifithrin - μ treatment compared to 24 h treatments (Fig 4.3.5 B), Table 4.3).

Treatment with pifithrin chloride also expressed cytotoxicity in U937 cell lines in a concentration dependent manner. 24 h treatment with pifithrin chloride displayed progressive cytotoxicity at concentrations starting 25μM with significance of P<0.0001 (Fig 4.3.7 A, Table 4.4) compared to untreated cell lines with MTS cell viability assay. PI staining assay confirmed necrotic effect with increase in drug dose showing significance of P<0.0001 between doses 25 μM and 50 μM (Fig 4.3.7 B) displaying significance of (P<0.0001***) Table 4.4), with a further rise in cell necrosis with concentration compared to pifithrin - μ treatments. pifithrin chloride inferred no change from 24 h MTS assay results and the results also stated reduced necrotic effect after 48h exposure in U937 cell lines (Fig 4.3.8 B, Table 4.5).

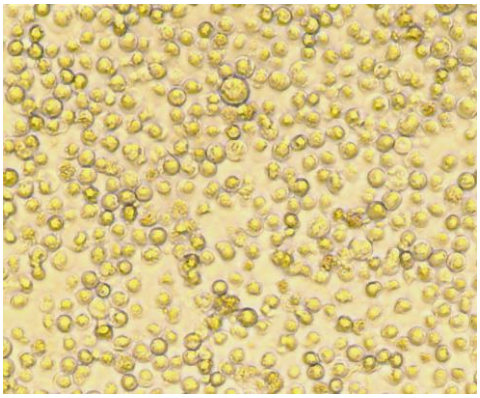
A



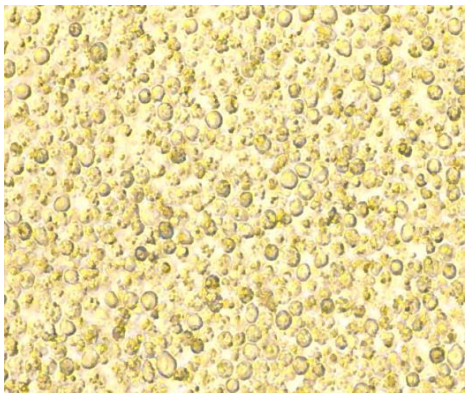
B



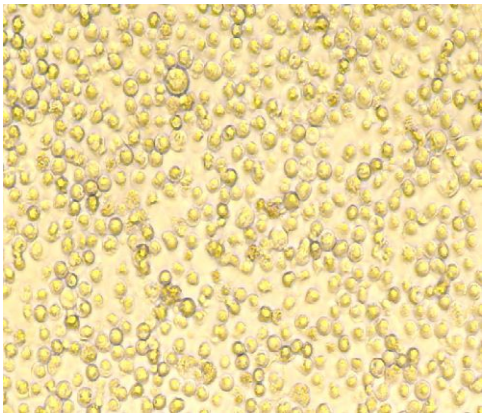
C



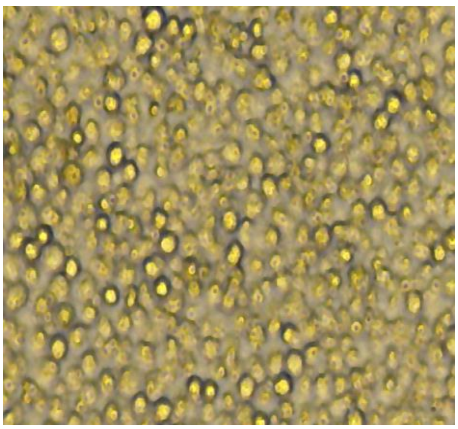
D



E



F



G

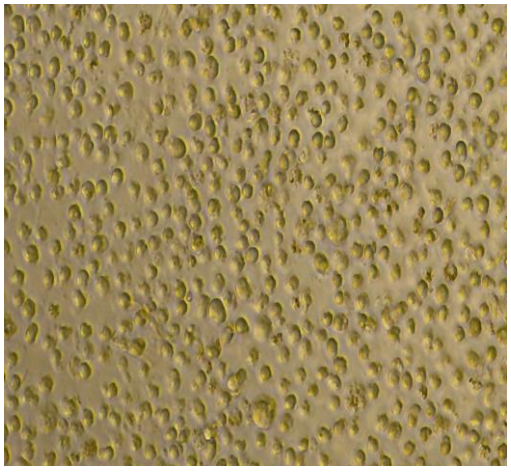


Fig: 4.3.3 Microscopic observation of U937 cell lines treated with pifithrin - μ at magnification of 20X objective (2048 × 1536 px).
The cells, following treatment and incubation for 24 h at 37°C, were microscopically observed for cytotoxicity; (A) untreated, pifithrin-μ- (B) 25μM, (C) 50μM, (D) 100μM; Pifithrin chloride treated – (E) 25μM, (F) 50μM and (G) 100μM.

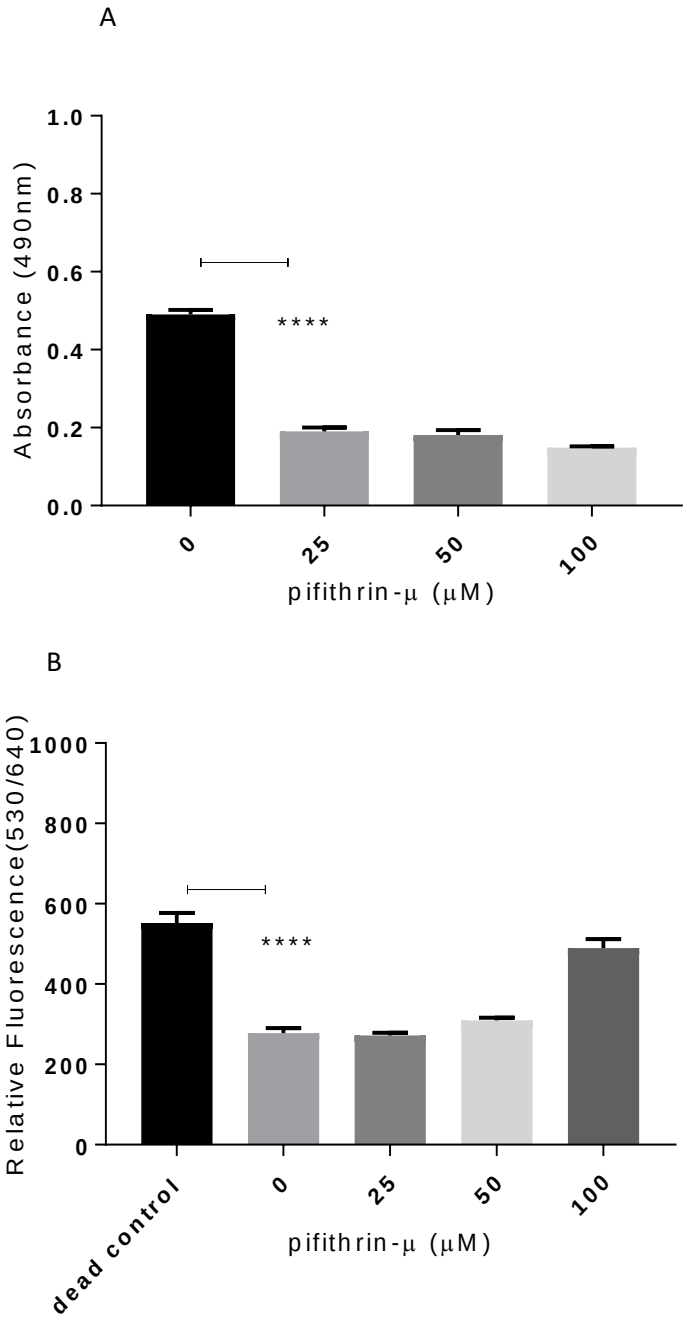


Fig: 4.3.4: **Effect on cell viability by pifithrin - μ on U937 cell lines.** U937 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) were treated with varying concentrations of pifithrin - μ (0,25,50,100μM). The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin - μ concentrations are indicated by **** (p<0.05 mean±SD; n=4,).

Table4. 2 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			PI Assay		
Pifithrin -μ(μM)	**Significance**	**P value**	**Pifithrin -μ(μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	ns	P 0.9875
25 vs 50	ns	P 0.6374	25 vs 50	*	P 0.0463
50 vs 100	**	P 0.0040	50 vs 100	****	P<0.0001

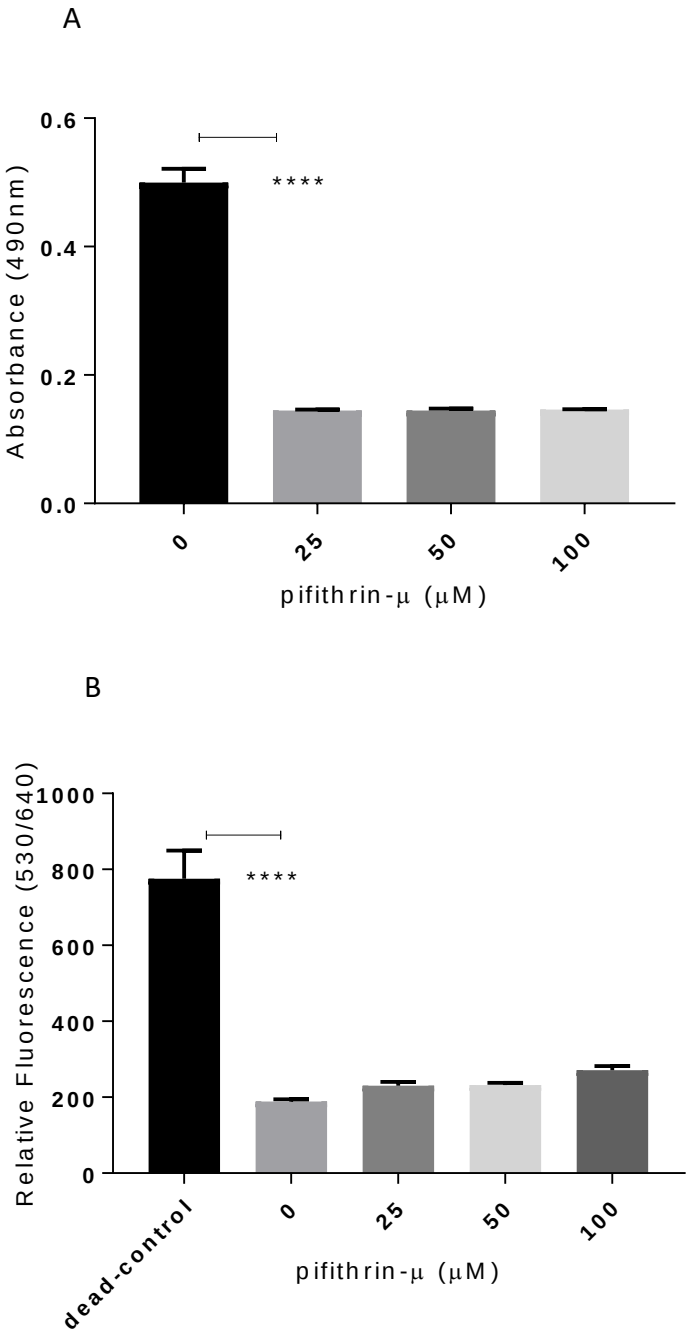


Fig: 4.3.5: **Effect on cell viability by pifithrin - μ on U937 cell lines.** U937 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) were treated with varying concentrations of pifithrin - μ (0,25,50,100μM). The cells were allowed for 48 h in incubation at 37°C: cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and Pifithrin - μ concentrations are indicated by **** ($p<0.05$, mean±SD; n=4).

Table 4.3 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin -μ(μM)	**Significance**	**P value**	**pifithrin -μ(μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	ns	P 0.3032
25 vs 50	ns	P>0.9999	25 vs 50	ns	P>0.999
50 vs 100	ns	P 0.9970	50 vs 100	ns	P 0.3700

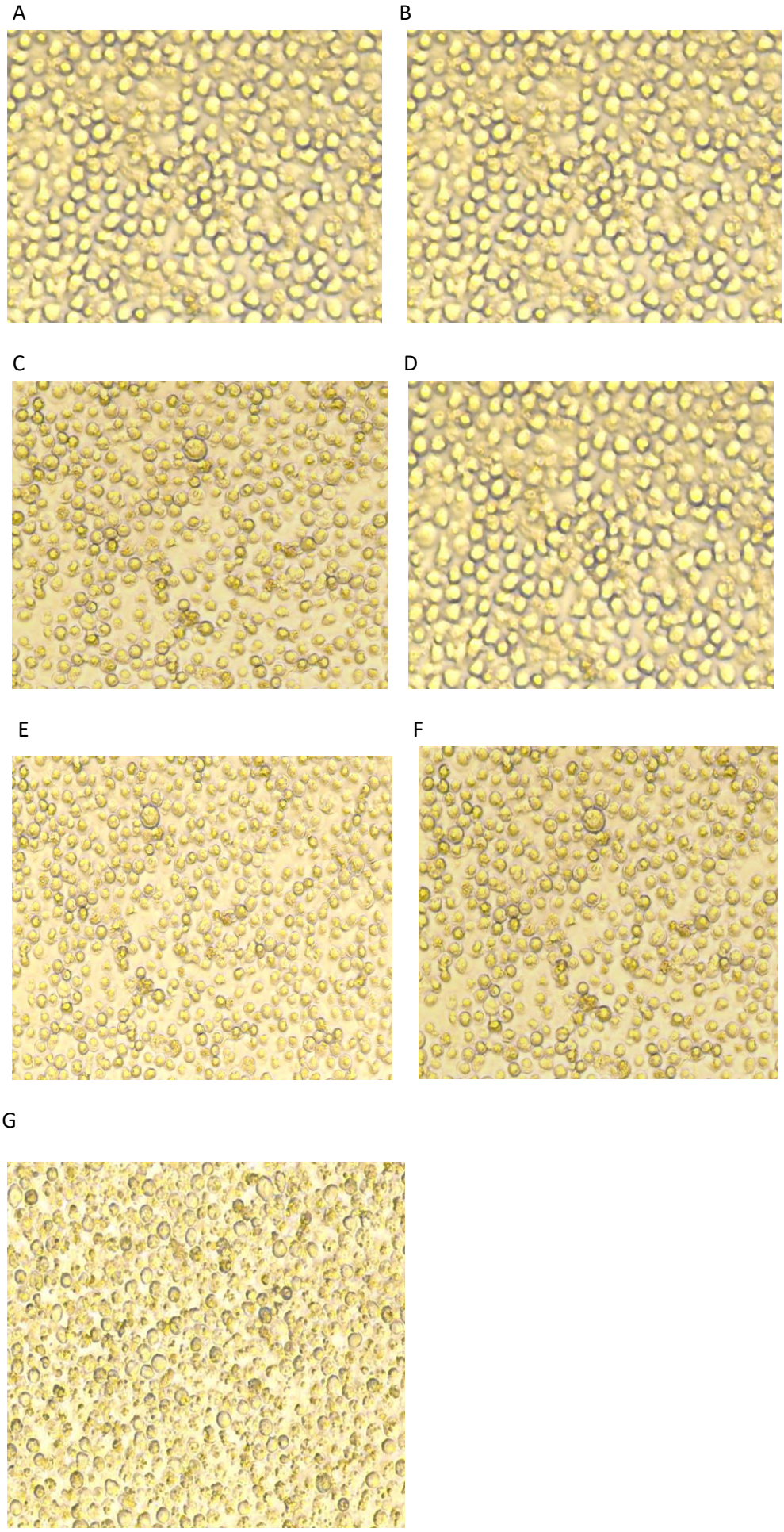


Fig: 4.3.6 Microscopic observation of U937 cell lines treated with pifithrin - μ, pifithrin chloride at magnification of 20X objective (2048 × 1536 px). Cells following treatment and incubation for 24 h at 37℃, were microscopically observed for cytotoxicity; (A) untreated, pifithrin-μ- (B) 25μM, (C) 50μM, (D) 100μM; Pifithrin chloride treated – (E) 25μM, (F) 50μM and (G) 100μM.

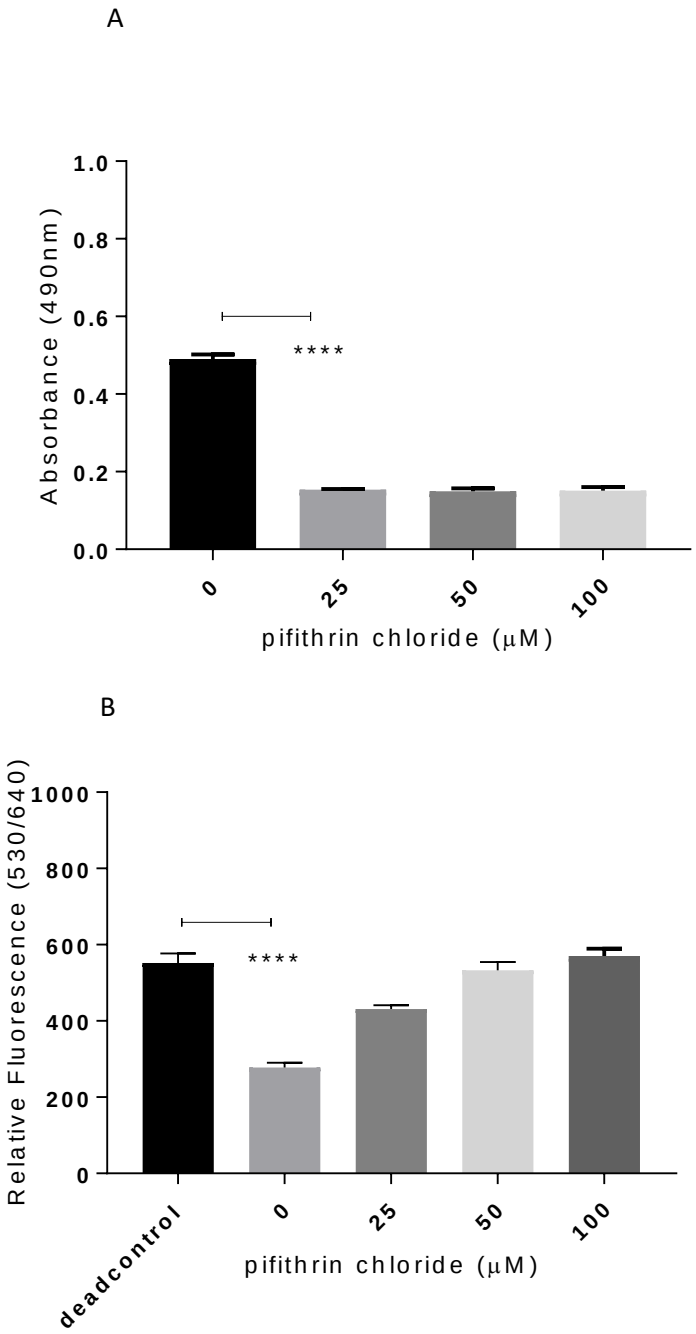


Fig: 4.3.7: **Effect on cell viability by pifithrin chloride on U937 cell lines.** U937 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) were treated with varying concentrations of pifithrin chloride (0,25,50,100μM). The cells were allowed for 24h in incubation at 37℃. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one- way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin chloride concentrations are indicated by **** ($p<0.05$) mean±SD; n=4).

Table 4.4 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin chloride(μM)	**Significance**	**P value**	**pifithrin chloride(μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	****	P<0.0001
25 vs 50	ns	P 0.9012	25 vs 50	****	P<0.0001
50 vs 100	ns	P 0.9830	50 vs 100	ns	P 0.0780

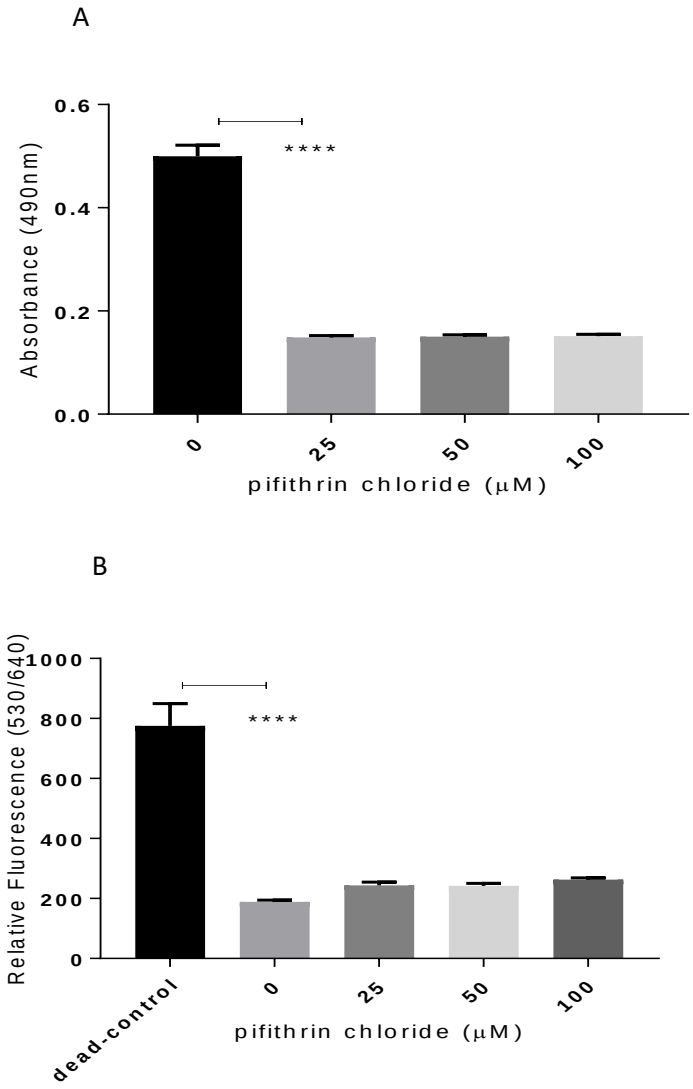


Fig: 4.3.8 Effect on cell viability by pifithrin chloride on U937 cell lines. U937 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) were treated with varying concentrations of pifithrin chloride (0,25,50,100μM). The cells were allowed for 48 h incubation at 37℃. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one- way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin chloride concentrations are indicated by **** ($p<0.05$ mean±SD; n=4).

Table 4.5 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin chloride (μM)	**Significance**	**P value**	**pifithrin chloride (μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	ns	P 0.1081
25 vs 50	ns	P 0.9957	25 vs 50	ns	P >0.999
50 vs 100	ns	P 0.9837	50 vs 100	ns	P 0.835

4.3.3 Effect of pifithrin - μ (20μM) and (10μM) combined with low UV radiation on U937 cell line

The experiments followed were designed to determine the effect of UV radiation exposure 2 s and 4 s in combination with pifithrin - μ at concentrations of 20 μM and 10 μM. pifithrin - μ only expressed significant drop in viability at 20 μM (P<0.0001****) by MTS assay in combination with UV (Fig 4.3.10A, Table 4.6). Even so no necrosis was observed with any of the UV - pifithrin - μ combination treatments (Fig 4.3.10 B, Table 4.6). No significant difference in the cell viability was observed in this combination treatment as pifithrin - μ at 20 μM itself produced considerable cytotoxicity at this tested concentration. Hence, the treatment was repeated with reduced doses of pifithrin -μ (10μM) together with low intensity UV irradiation to reveal whether it could present a progressive cytotoxic activity with response to UV exposure.

Though pifithrin - μ (10 μM) expressed insignificant difference in cell viability (P 0.8991) in contrast to untreated controls by microscopic observation (Fig 4.3.9) or MTS assay that presented significant difference in combination with UV exposure at 2 s (P<0.0001****) and 4 s (P <0.0001***, Fig 4.3.11A, Table 4.7). The PI assay expressed insignificant variation between pifithrin - μ - UV treated groups at 2 s (P 0.8708) and 4 s (P 0.9115) respectively (Fig 4.3.10B).

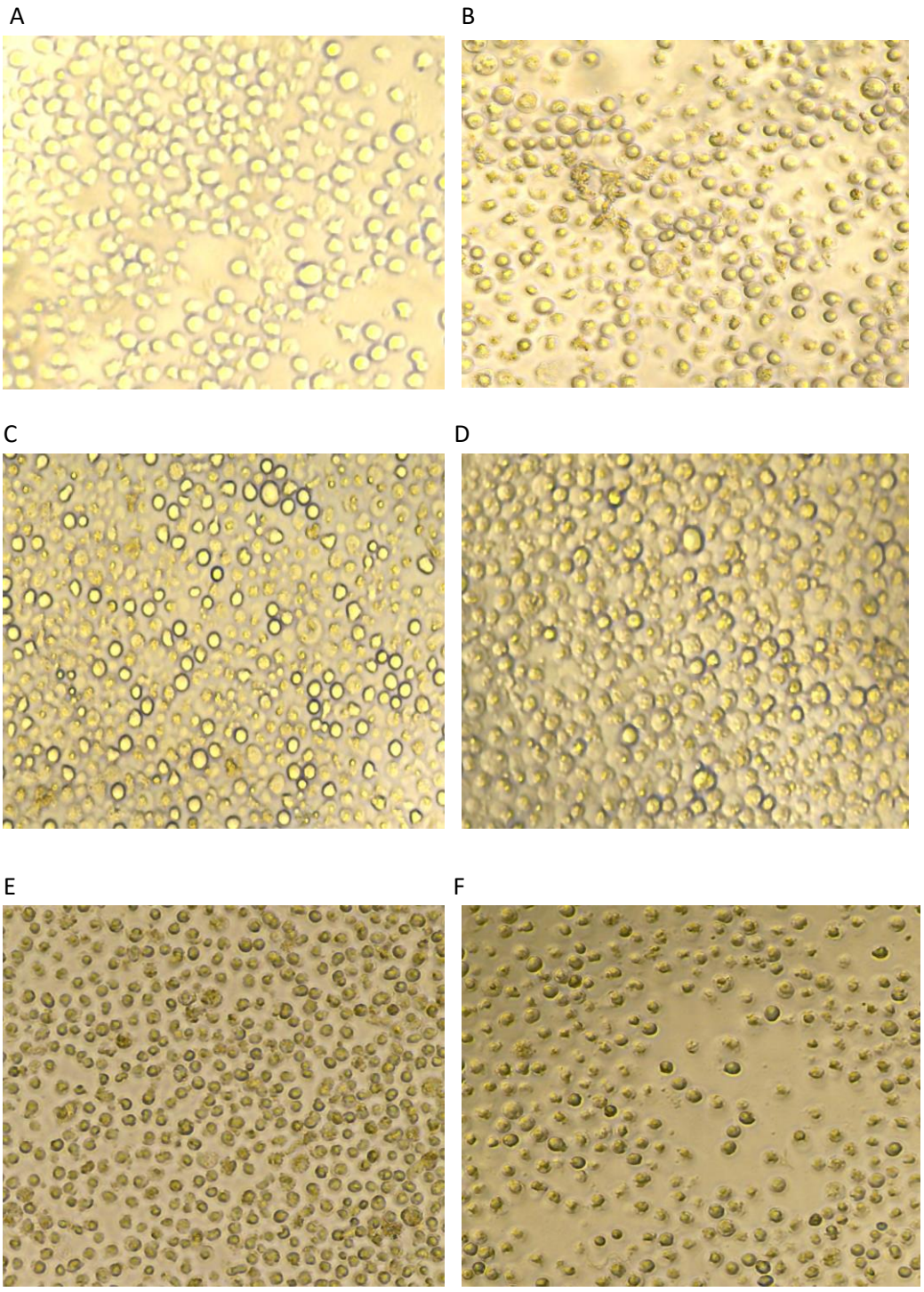


Fig: 4.3.9 Microscopic observation of U937 cell lines treated with pifithrin-μ and UV radiation at magnification of 20X objective (2048 × 1536 px). U937 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) were treated with Pifithrin-μ (10 μM) I h prior to UV exposure for 2 and 4 s , were microscopically observed for cytotoxicity; (A) untreated, (B) pifithrin-μ (10 μM) (C) UV 2(s), (D) UV 4(s), (E) pifithrin-μ- UV 2(s), (F) pifithrin-μ- UV 4(s).

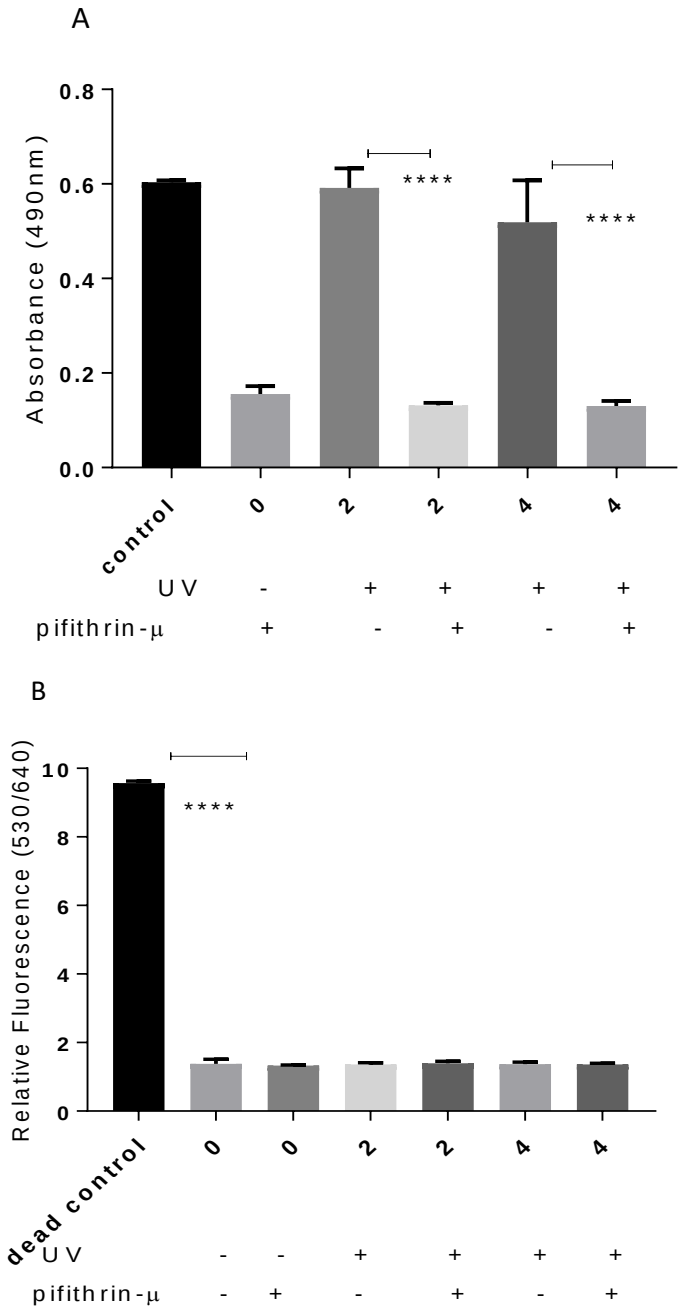


Fig: 4.3.10 Effect on cell viability by pifithrin- μ(20μM) with low UV on U937 cell line. U937 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) were treated with pifithrin-μ (20 μM) for 1h prior to UV exposure for 2 and 4 s, Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one- way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between UV and Pifithrin- μ - UV concentrations are indicated by **** ($p<0.05$, mean±SD; n=4).

Table 4.6 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
UV exposure (s) / pifithrin - μ (μM)	**Significance**	**P value**	**UV exposure (s) / pifithrin -μ (μM)**	**Significance**	**P value**
Pifithrin (-) UV 2(+) vs UV 2 (+) pifithrin (+)	****	P <0.0001	pifithrin (+) UV 2(-) vs UV 2 (+) pifithrin (+)	ns	P 0.9952
pifithrin (-) UV 4(+) vs UV 4 (+) pifithrin (+)	****	P <0.0001	pifithrin (+) UV 4(-) vs UV 4 (+) pifithrin (+)	ns	P >0.9999
pifithrin (-) UV 2(-) vs UV 2 (+) pifithrin (-)	ns	P 0.9983	pifithrin (-) UV 2(-) vs UV 2 (+) pifithrin (-)	ns	P >0.9999
pifithrin (-) UV 4(-) vs UV 4 (+) pifithrin (-)	ns	P 0.0790	pifithrin (-) UV 4(-) vs UV 4 (+) pifithrin (-)	ns	P >0.9999

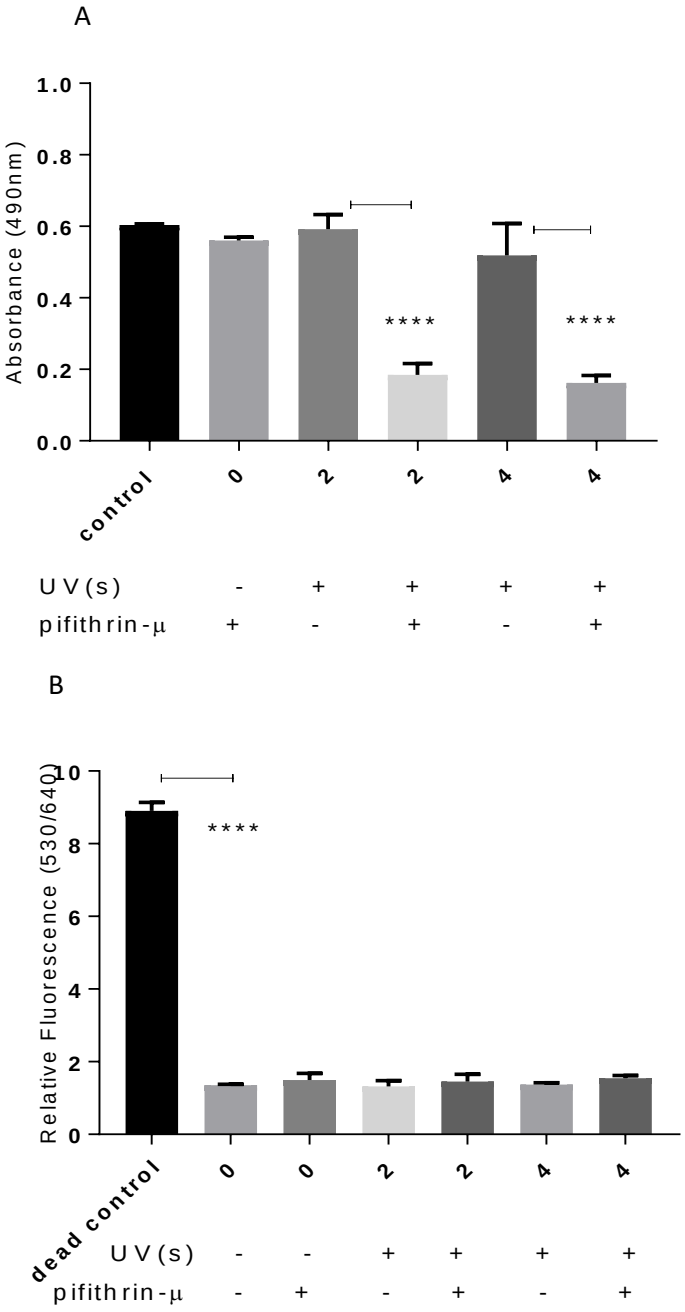


Fig: 4.3.11 Effect on cell viability by pifithrin μ(10μM) combined with UV on U937 cell line U937 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) were treated with pifithrin - μ (10 μM) 1 h prior to UV exposure for 2 and 4 s. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between UV and Pifithrin- μ - UV treatments are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 4.7 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
UV exposure(s) Piffithrin - μ (μM)	**Significance**	**P value**	**UVexposure(s)/Piffithrin - μ (μM)**	**Significance**	**P value**
pifithrin (-) UV 2(+) vs UV 2 (+) pifithrin (+)	****	P<0.0001	pifithrin (+) UV 2(-) vs UV 2 (+ pifithrin (+)	ns	P 0.8708
pifithrin (-) UV 4(+) vs UV 4 (+) pifithrin (+)	****	P<0.0001	pifithrin (+) UV 4(-) vs UV 4 (+) pifithrin (+)	ns	P 0.9115
pifithrin (-) UV 2(-) vs UV 2 (+) pifithrin (-)	ns	P 0.9987	pifithrin (-) UV 2(-) vs UV 2 (+) pifithrin (-)	ns	P 0.6747
pifithrin (-) UV 4(-) vs UV 4 (+) pifithrin (-)	ns	P 0.1050	pifithrin (-) UV 4(-) vs UV 4 (+) pifithrin (-)	ns	P 0.9115

4.3.4 Effect of hyperthermia 42◦C on U937 cell lines

Hyperthermia induced a significant drop in cell viability on U937 cell lines exposed to 42℃ treatment (P 0.0025**, Fig:4.3.12 A, Table 4.8) with respect to the untreated controls. However, no necrosis was detected by PI (P 0.0883) at 42℃ or 37℃ treatment (Fig:4.3.12 B, Table 4.8).

4.3.5 Determination of hyperthermia induced apoptosis by flow cytometry

The cytotoxic activity induced by heat shock treatment in U937 cell line were confirmed by performing annexin V and PI double staining. The flow cytometry data (Fig:4.3.13 C) expressed a viable population of 92.4% in the untreated controls. Heat shock treatment displayed decline in viable population around 38% due to 42℃ heat shock exposure (Fig:4.3.13 A). The data also exhibited significant early apoptotic rate of 17.9% (P 0.0219**, Fig:4.3.13 B), 23.5% of the population directed towards late apoptosis (P 0.0257**, Fig:4.3.13 D) and confined cells 4.4% undergoing necrosis that were displayed insignificant (P 0.0914, Fig:4.3.13 C) in contrast to untreated cells at 37℃. Hence the results stated that hyperthermia induced cytotoxicity is resulted due to apoptotic cell death.

4.3.6 Hyperthermia and HSPA1A protein in U937 cell lines

The U937 cell line exposed to hyperthermia treatment at 42℃ resulted in increased surface release of HSPA1A. HSPA1A expression was measured by flow cytometry using FITC - labelled secondary antibody as presented in (Fig 4.3.15). Heat treatment resulted in significant surface release of HSPA1A proteins at a rate of around 38.6% (P 0.0002***), which was comparatively higher with that of the untreated cells, which also expressed HSPA1A (18.6%) (Fig:4.3.15 D).

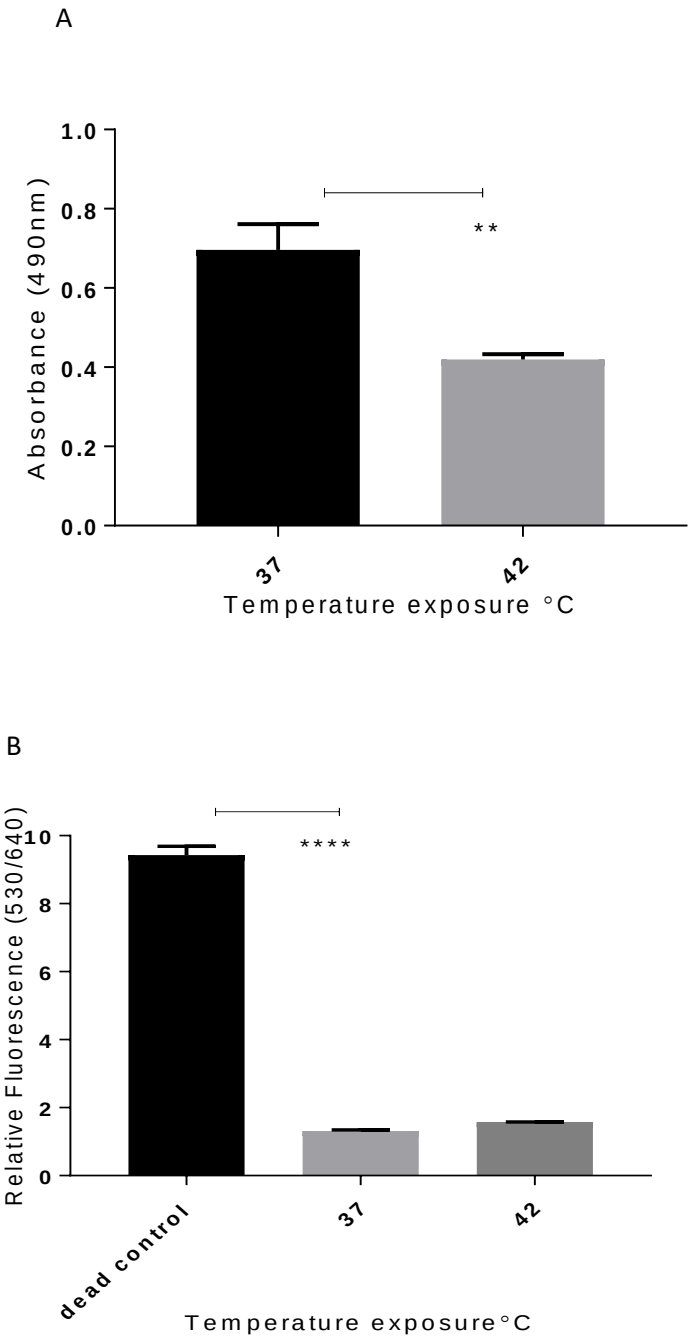
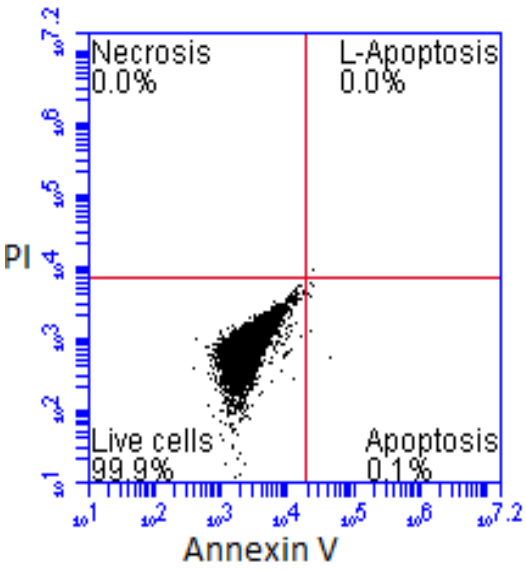


Fig: 4.3.12 Effect on cell viability by hyperthermia - 42℃ on U937 cell lines. U937 cell lines at cell density (5 × 10^5(cells/ml), 3ml of cells were subjected to heat shock treatment at 42◦C for 1 h, followed by 2 h recovery at 37◦C. The cells were allowed for incubation at 37◦C for 24 h. The cells were then analyzed by(A) MTS or (B) PI. Data were analyzed statistically using (A) paired t test and (B) one- way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between temperature treatments are indicated by **** ($p<0.05$ mean±SD; n=4).

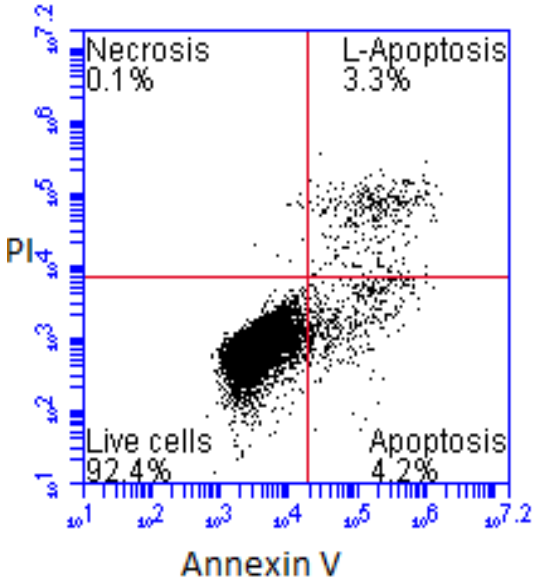
Table 4.8 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			PI Assay		
Temperature℃	**Significance**	**P value**	**Temperature℃**	**Significance**	**P value**
37 vs 42	**	P 0.0025	37 vs 42	ns	P 0.0883

A Unstained control



B 37°C



C 42°C Heat shock

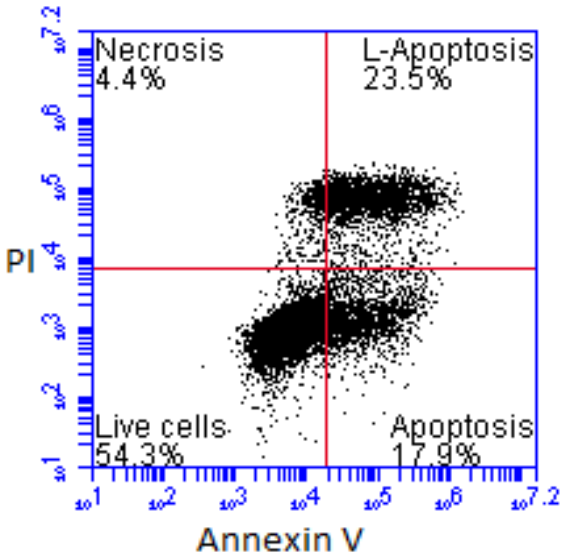


Fig: 4.3.13 Flowcytometric data presentation of annexin V and PI staining in U937 cell lines exposed to 42 °C heat shock treatment. U937 cell lines at cell density (1×10^6 cells), aliquots of 3 ml cells were subjected to heat shock treatment at 42°C for 1 h, followed by 2 h recovery at 37°C. The cells were allowed for incubation at 37°C for 24 h. The cells were then analyzed by annexin V and PI staining using flow cytometer (A) unstained control, (B) 37°C and (C) 42°C heat shock treated.

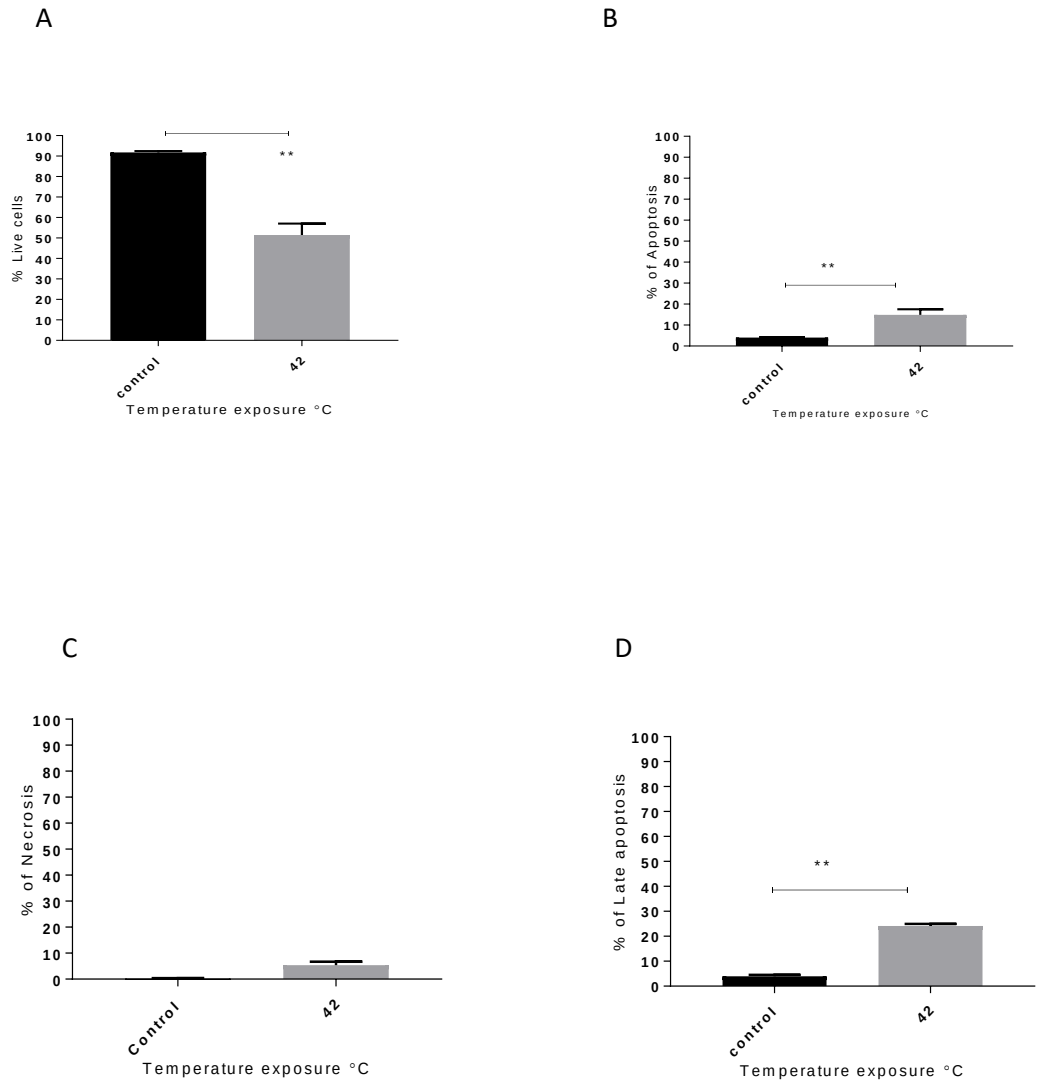


Fig: 4.3.14 Data interpretation of annexin V and PI staining in U937 cell lines exposed to 42 ℃ heat shock treatment. U937 cell lines at cell density (1 × 10^6 cells), aliquots of 3 ml cells were subjected to heat shock treatment at 42℃ for 1 h, followed by 2 h recovery at 37℃. The cells were allowed for incubation at 37℃ for 24 h. The cells were then analyzed by annexin V and PI staining using flow cytometer. Data were analyzed statistically using paired t test and significant differences between temperature treatments are indicated by * ($p<0.05$) n=3; (A) 37 vs 42 (P 0.0085**) (B) (P-0.0219**) significant; (C) P 0.0914) insignificant;(D) (P 0.0257**).

.

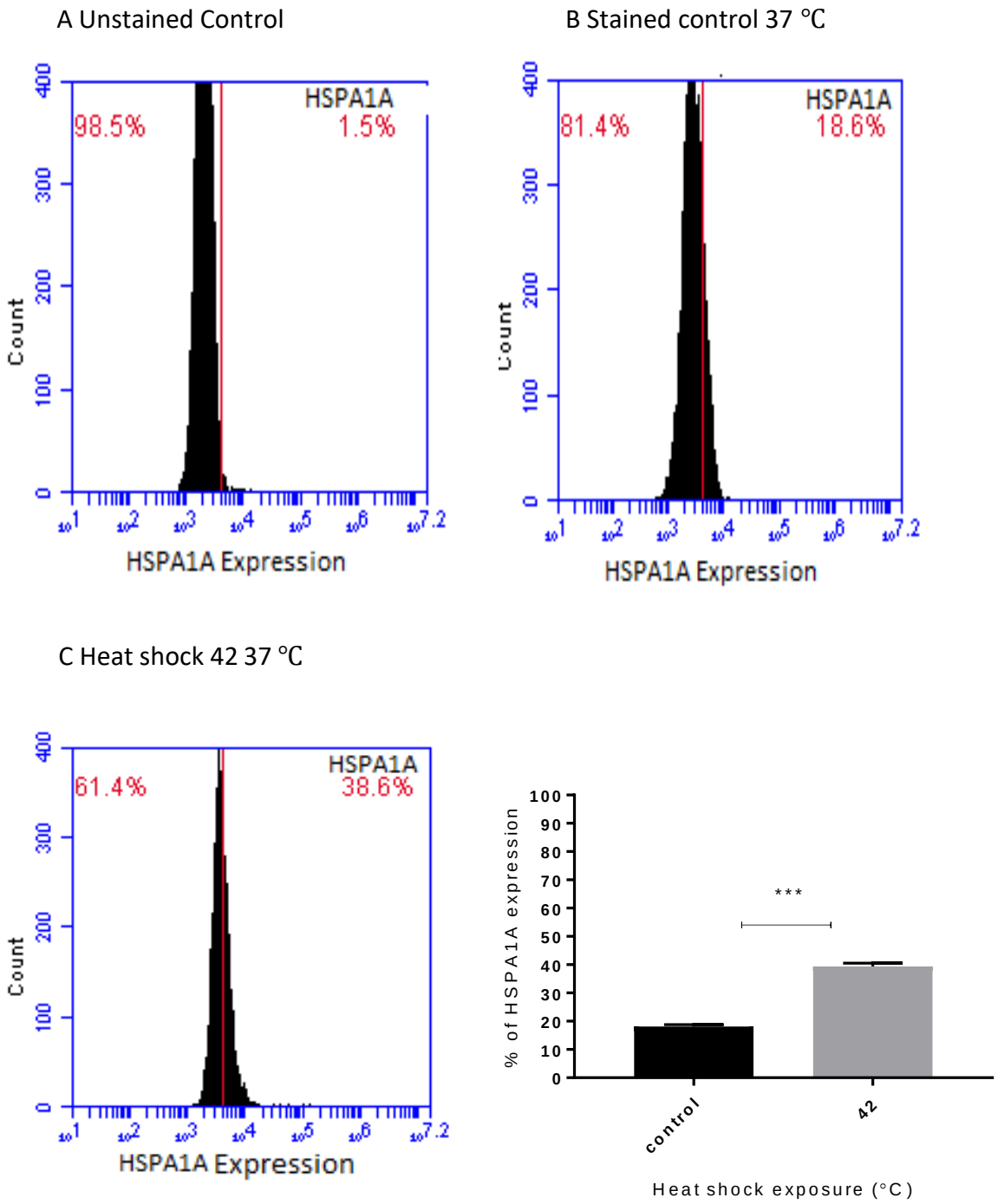


Fig: 4.3.15 Expression of cell surface HSPA1A in U937 leukaemia cell lines exposed to 42 ℃ heat shock treatment. U937 cell lines at cell density (1 × 10^6 cells), aliquots of 3 ml cells were subjected to heat shock treatment at 42℃ for 1 h, followed by 2 h recovery at 37℃. The cells were allowed for incubation at 37℃ for 24 h. The cells were then analyzed for HSPA1A expression using FITC labelled secondary antibodies using flow cytometer. Flow cytometry data presentation of histograms gated with (A) primary antibody stained control); (B 37℃); (C) 42 ℃ heat treatment (D) Data were analyzed statistically using paired-t test represented mean±SD(P<0.05) n=3; control vs 42℃ treatment (p-0.0105***) mean±SD; n=3.

4.3.7 Effect of pifithrin - μ and pifithrin chloride in HT-29 colorectal cancer cell lines

The HT-29 cancer cell lines were treated with pifithrin - μ at concentrations ranging 25,50 and 100μM allowed for incubation for 24 h. MTS cell viability assay which exhibited cytotoxicity at all the treated concentrations (Fig:4.3.16 A) with insignificant variations between concentrations -25 μM vs 50 μM (P 0.9774);100 (P >0.9999, Table 4.9). PI assay presented necrosis with increase in concentration with significant difference between different treated concentrations (Fig:4.3.16 B) as presented in (Table 4.9). Upon continuing pifithrin - μ treatment for 48 h, MTS results showed same effect as depicted at 24 h (Fig:4.3.17 A), while the necrotic effect declined considerably, displaying insignificant difference between the tested concentrations as presented in (Fig:4.3.17 B, Table 4.10).

The HT-29 cancer cell lines were found more effective upon treatment with Pifithrin chloride compared to pifithrin - μ. pifithrin chloride were tested at concentrations ranging 25,50 and 100μM/ml and allowed for incubation for 24 hours. MTS cell viability assay and PI assay displayed a progressive cytotoxic activity at all the treated concentrations (Fig 4.3.18A) in contrast to the untreated control, while there exhibited no significance difference between treated dose,25 vs 50 (P 0.9988); 50 vs 100(P 0.9994) (Table 4.11). PI staining assay revealed necrotic effect with significant variation between 25 vs 50 concentrations (P 0.0155*) (Fig:4.3.18 B) and was found insignificant with higher concentration of 100μM (P 0.2611). Upon continuing pifithrin chloride treatment for 48 h, MTS results remained same as exhibited at 24 h (Fig:4.3.19 A), while the necrotic effect declined considerably with insignificant variation between tested doses as displayed in (Fig:4.3.19 B, Table 4.12).

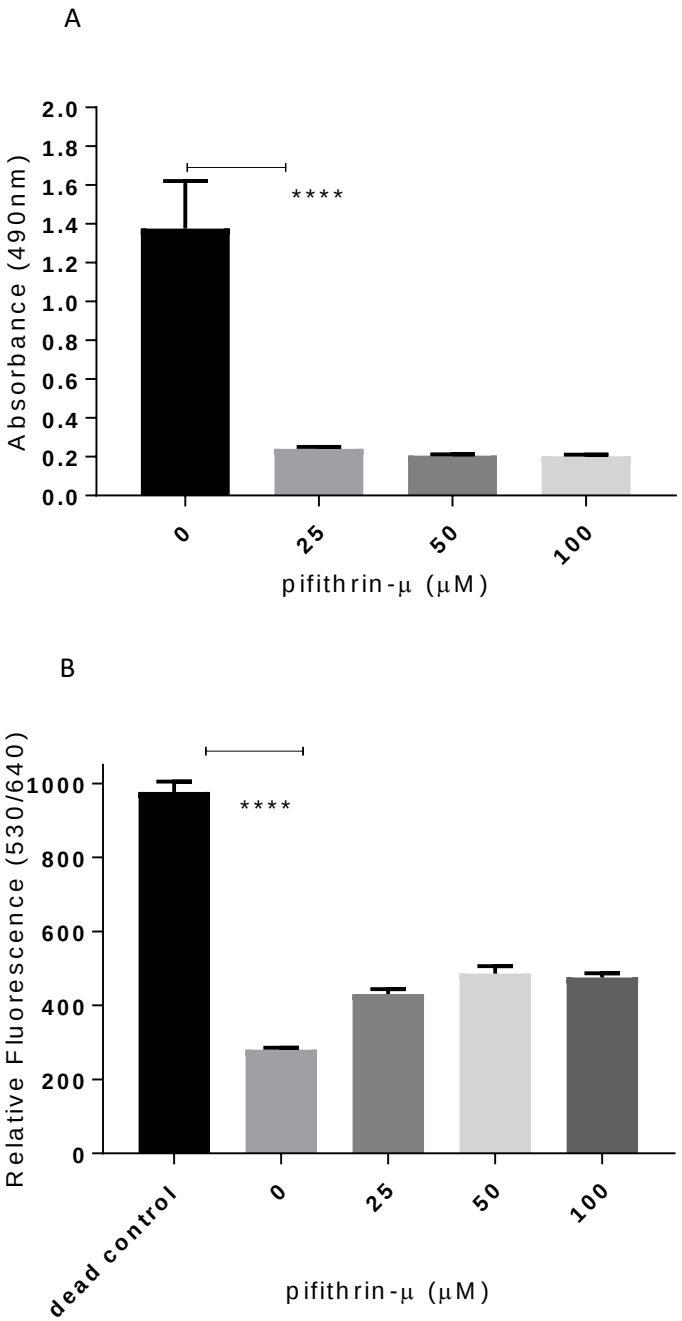


Fig: 4.3.16 Effect on cell viability by pifithrin μ on HT-29 cell lines for 24h. HT-29 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with varying concentrations of pifithrin - μ (0,25,50,100μM). The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin - μ concentrations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 4.9 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin -μ (μM)	**Significance**	**P value**	**pifithrin-μ (μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	****	P<0.0001
25 vs 50	ns	P 0.9774	25 vs 50	**	P 0.0032
50 vs 100	ns	P>0.9999	50 vs 100	**	P 0.8926

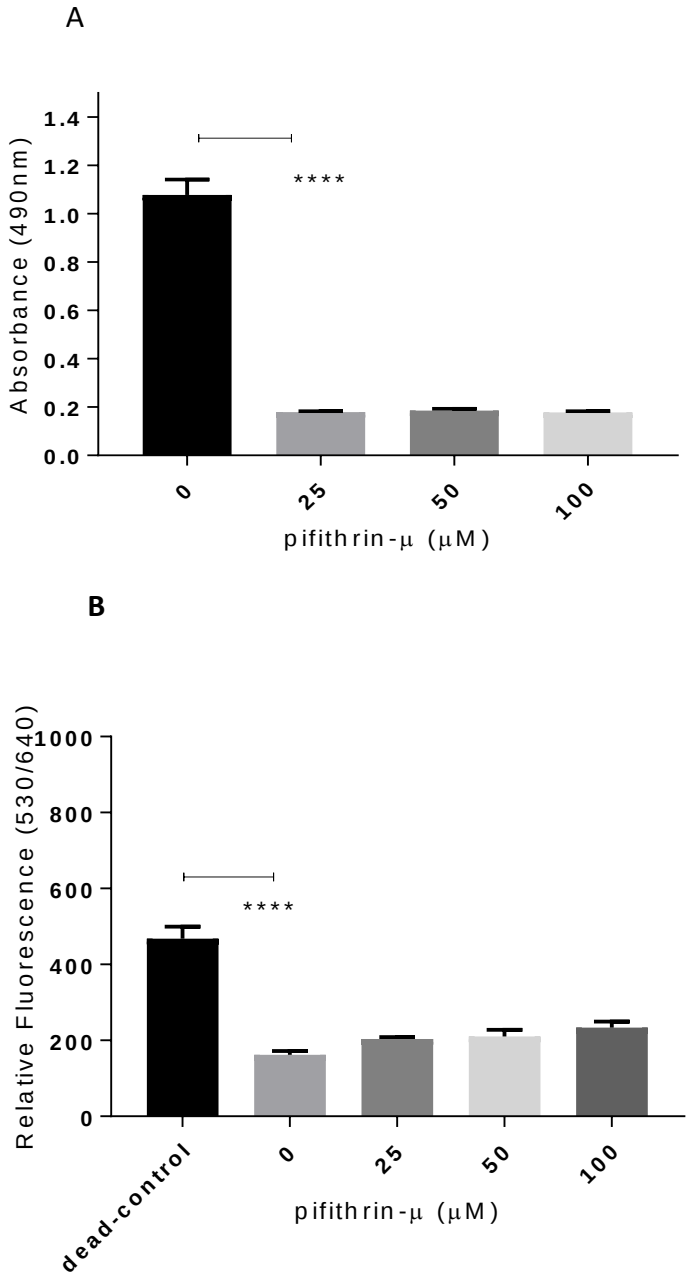


Fig: 4.3.17 Effect on cell viability by pifithrin - μ on HT-29 cell lines for 48 h. HT-29 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with varying concentrations of Pifithrin-μ (0,25,50,100μM). The cells were allowed for 48 h in incubation at 37°C. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin - μ concentrations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 4.10 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin -μ (μM)	**Significance**	**P value**	**pifithrin -μ (μM)**	**Significance**	**P value**
0 vs 25	****	P< 0.0001	0 vs 25	*	P 0.0393
25 vs 50	ns	P 0.9903	25 vs 50	ns	P 0.9882
50 vs 100	ns	P>0.9856	50 vs 100	ns	P 0.3894

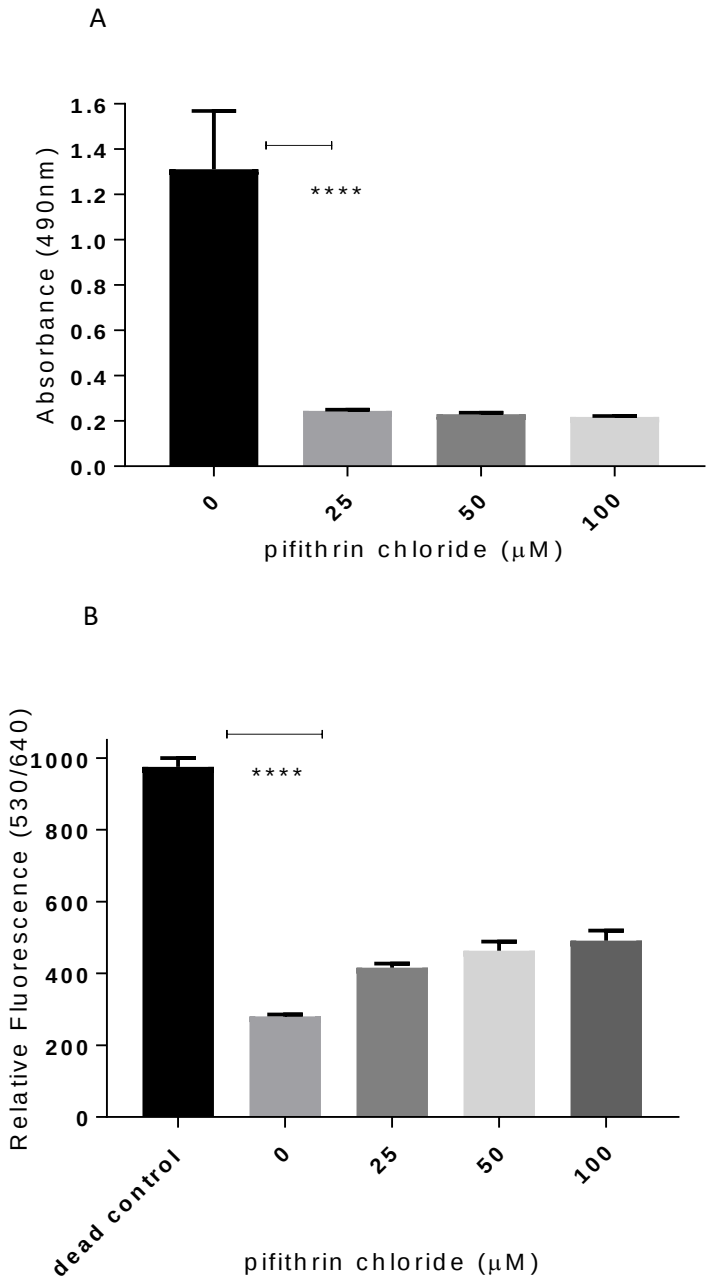


Fig: 4.3.18 Effect on cell viability by pifithrin chloride on HT-29 cell lines for 24h. HT-29 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with varying concentrations of pifithrin chloride (0,25,50,100μM). The cells were allowed for 24 h incubation at 37℃. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin chloride concentrations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 4.11 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin chloride(μM)	**Significance**	**P value**	**pifithrin chloride(μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	****	P<0.0001
25 vs 50	ns	P 0.9988	25 vs 50	*	P 0.0155
50 vs 100	ns	P 0.9994	50 vs 100	ns	P 0.2611

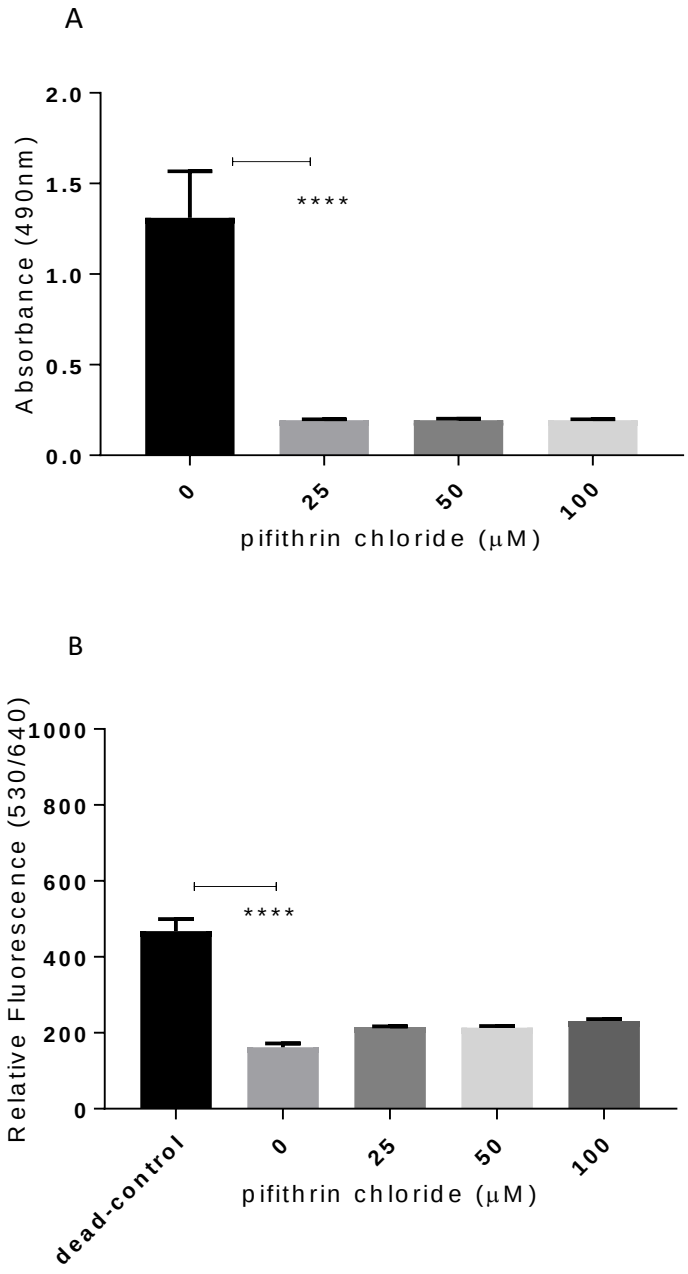


Fig: 4.3.19 Effect on cell viability by pifithrin chloride on HT-29 cell lines for (48 h). HT-29 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with varying concentrations of pifithrin chloride (0,25,50,100μM). The cells were allowed for 48 h incubation at 37°C. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between untreated (0) and pifithrin chloride concentrations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 4.12 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay			**PI Assay**		
pifithrin chloride(μM)	**Significance**	**P value**	**pifithrin chloride(μM)**	**Significance**	**P value**
0 vs 25	****	P<0.0001	0 vs 25	**	P 0.0013
25 vs 50	ns	P 0.9999	25 vs 50	ns	P 0.9999
50 vs 100	ns	P 0.9999	50 vs 100	ns	P 0.5107

4.3.8 Effect of pifithrin - μ combined with UV (4 s) exposure on HT-29 cell line

The colorectal cancer cell line HT-29 subjected to treatment with pifithrin - μ at varying concentrations 5, 10 and 15μM and the same concentrations were tested in combination with UV exposure for 4 s and allowed for incubation for 24 h. Cells expressed a pronounced drop in cell viability upon combination treatment of pifithrin - μ with UV at 4 s exposure by MTS assay displaying significant difference between varying concentration of pifithrin - μ with UV 4 s - pifithrin - μ, displaying 5 μM (P <0.0001****), 10 μM (P <0.0001****), 15μM (P <0.0001****) (Fig 4.3.20 A, Table 4.13).

The PI assay expressed no significant variation in combination treatment with UV 4 s exposure in all the pifithrin - μ concentrations tested, displaying 5 μM (P 0.9999), 10 μM (P >0.9999), 15μM (P 0.9999) (Fig 4.3.20 B, Table 4.13). The results from the study states the positive response of UV irradiation together with pifithrin - μ even at concentrations as low as 5 μM (P <0.0001****), accelerated the rate of cytotoxicity HT-29 cancer cell lines compared to pifithrin - μ treatment independently.

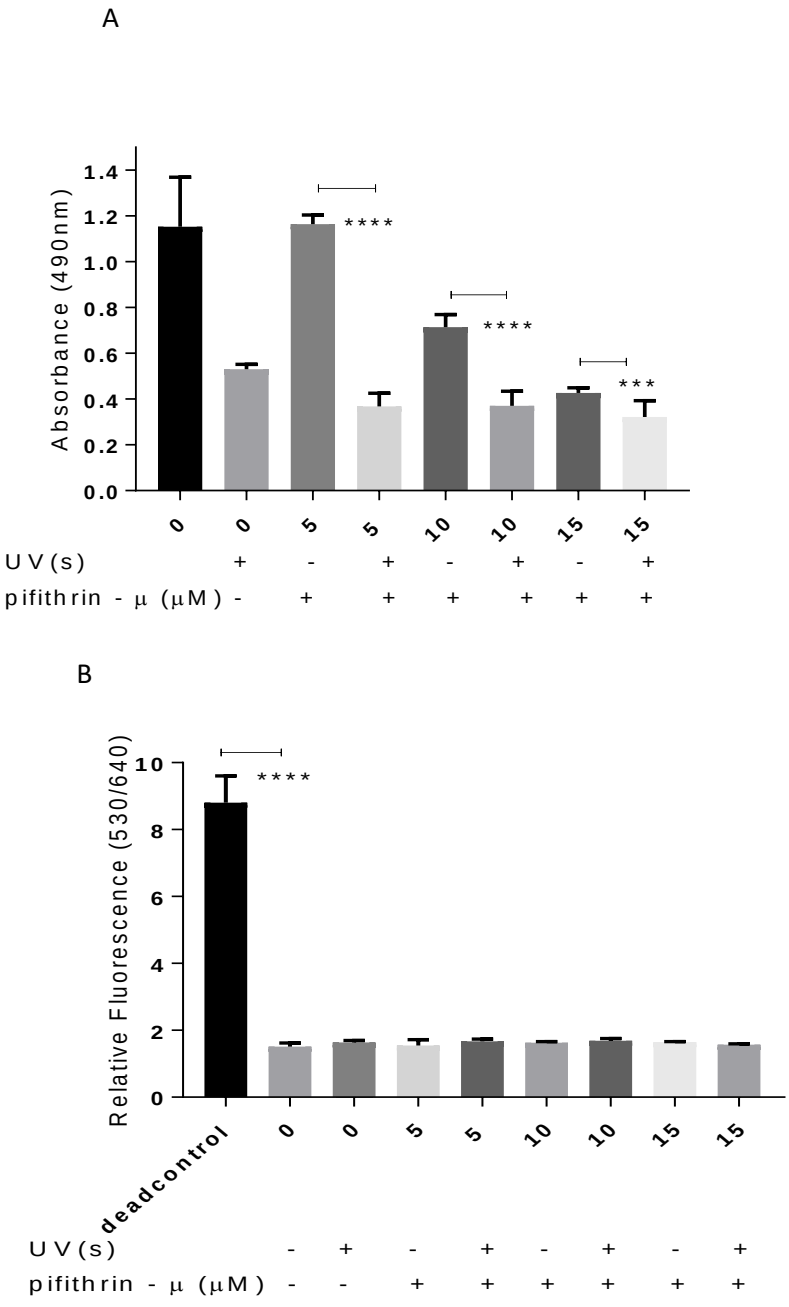


Fig: 4.3.20 Effect on cell viability by pifithrin - μ combined with UV (4s) exposure on HT-29 cell line. HT-29 cell lines seeded onto 96 well assay plates at cell density (5 × 10^5 cells/ml) were treated with pifithrin - μ (5,10, 15 μM) for 1 h prior to UV exposure for 4 s. The pifithrin -μ treatments independently were placed as reference. The cells were allowed for 24 h incubation at 37°C . Cell viability was measured by (A) MTS or (B) PI. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between UV and pifithrin - μ -UV concentrations are indicated by **** (p<0.05) mean±SD; n=4.

Table 4.13 Statistic presentation of Sidaks multiple comparisons test (ns* insignificant)

MTS Assay		
pifithrin-μ (μM)/UV(4s)	**Significance**	**P value**
pifithrin 5 (+) UV (-) vs UV (+) pifithrin 5 (+)	****	P<0.0001
pifithrin 10 (+) UV (-) vs UV (+) pifithrin10 (+)	****	P<0.0001
pifithrin 15 (+) vs UV (-) vs UV (+) pifithrin15(+)	***	P 0.0009
pifithrin (-) UV (-) vs UV (+) pifithrin (-)	****	P<0.0001

PI Assay		
pifithrin-μ (μM)/UV(4s)	**Significance**	**P value**
pifithrin 5 (+) UV (-) vs UV (+) pifithrin 5 (+)	ns	P 0.9999
pifithrin 10 (+) UV (-) vs UV (+) pifithrin10 (+)	ns	P 0.9999
pifithrin 15 (+) vs UV (-) vs UV (+) pifithrin15(+)	ns	P 0.9999
pifithrin (-) UV (-) vs UV (+) pifithrin (-)	**ns**	P 0.9992

4.3.9 Effect of hyperthermia in HT-29 cancer cell lines

The HT-29 colorectal cancer cell lines subjected to hyperthermia treatment at 42℃ displayed significant drop in cell viability according to MTS assay (Fig 4.3.21 A, P-0.0349*). No necrosis was observed due to hyperthermia by PI assay (Fig 4.3.21 B).

4.3.10 Determination of hyperthermia induced apoptosis by flowcytometry

The cytotoxicity induced hyperthermia by cell viability assays was further confirmed for apoptosis by annexin V and PI staining using flow cytometer. Hyperthermia at 42℃ resulted in a decline in viability to 30% (Fig 4.3.23 C), of which the early apoptotic % was 4.1 with late apoptosis of 25.8% (P 0.0085**, Fig: 4.3.23D).

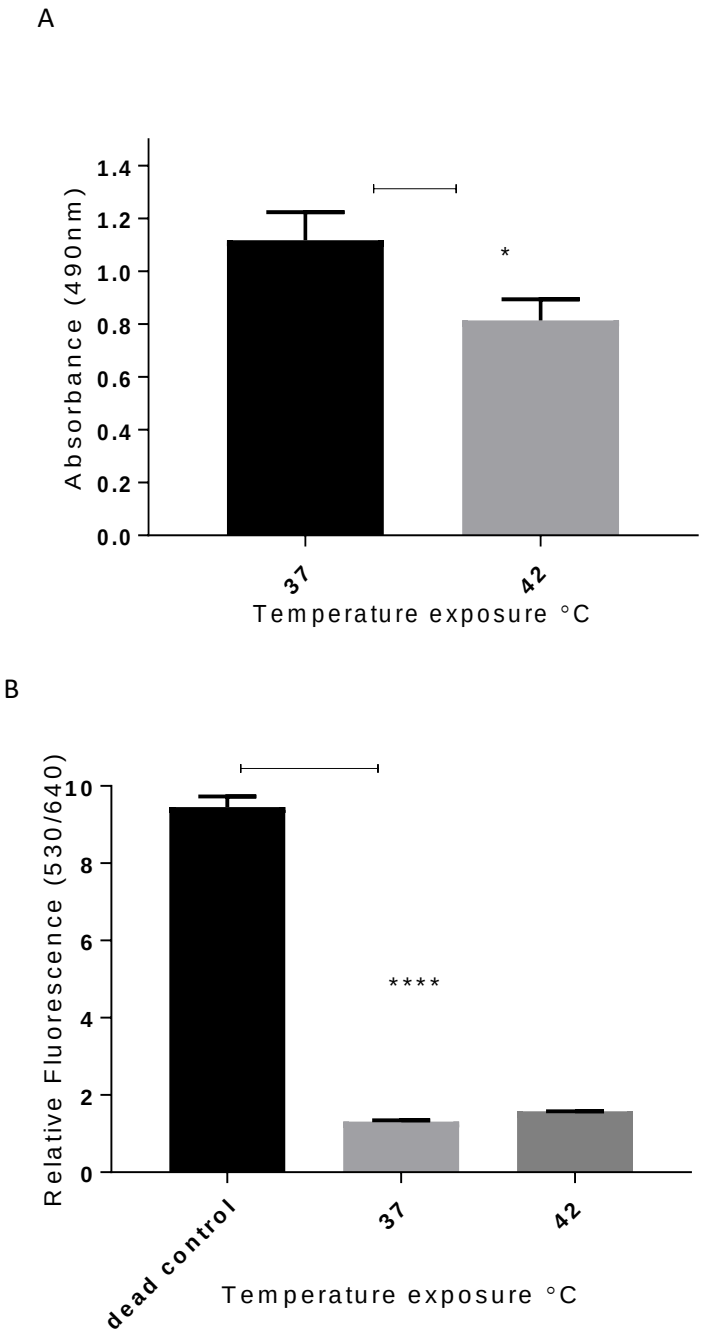


Fig: 4.3.21 Effect of heat shock exposure on HT-29 cell lines. U937 cell lines at cell density (5×10^5 cells/ml), aliquots of 3ml cells were subjected to heat shock treatment at 42◦C for 1 h, followed by 2 h recovery at 37◦C. The cells were allowed for incubation at 37◦C for 24 h. Cell viability was measured by (A) MTS or (B) PI. Data were analyzed (A) using paired t test, control vs 42 ℃ (P 0.0349*) (B) Using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between temperature treatments are indicated by ($p<0.05$) mean±SD; n=4 (37℃ vs 42℃ (P 0.0992).

4.3.10 Determination of hyperthermia induced apoptosis by flowcytometry

The apoptotic induced by hyperthermia at 42℃, examined by cell viability and PI assays were confirmed with annexin V and PI double staining in colorectal cancer cell line HT-29.

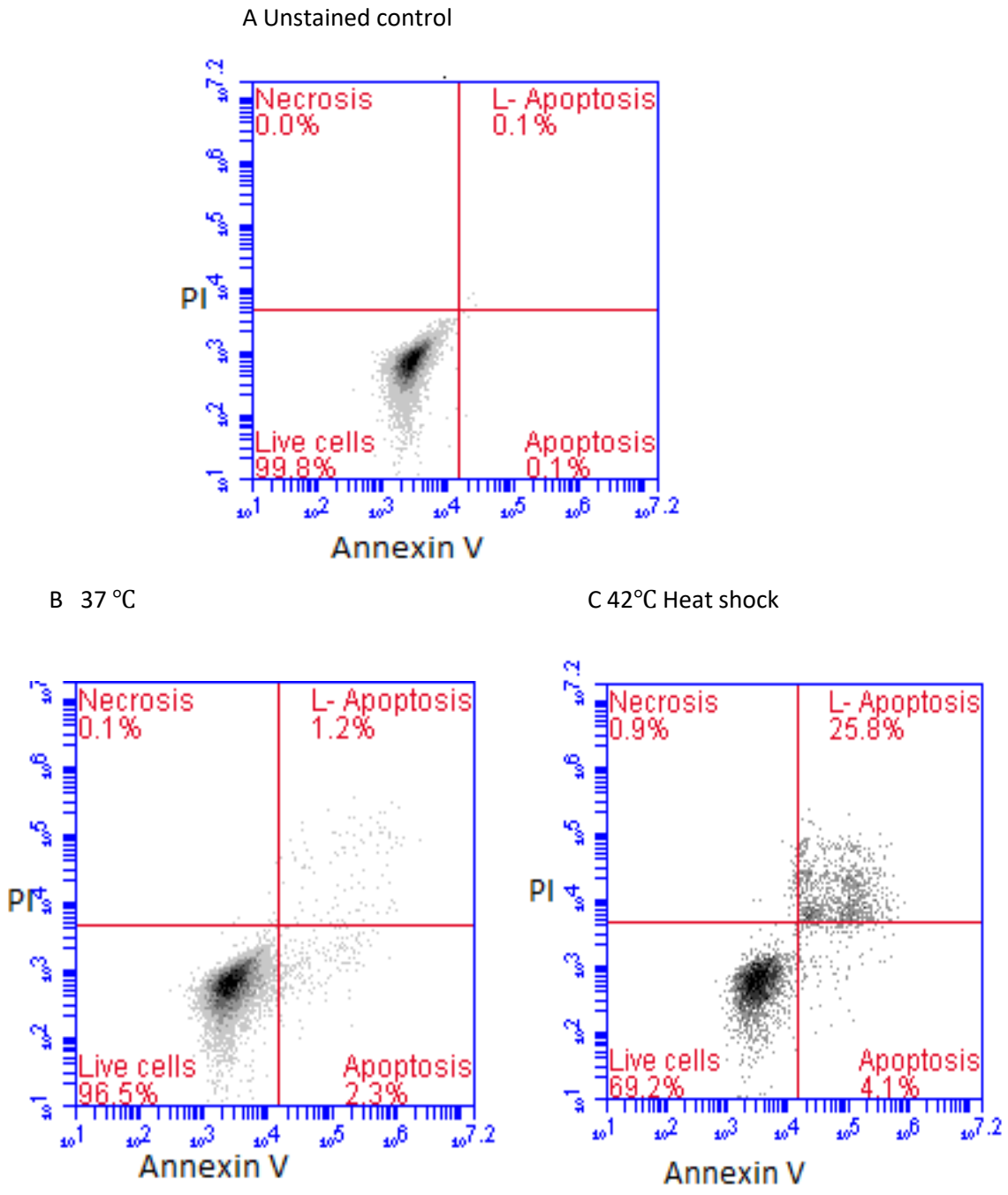


Fig: 4.3.22 Flowcytometric data presentation of annexin v and pi staining in HT-29 cell lines exposed to 42 ℃ heat shock treatment. HT-29 cell lines at cell density (1×10^6 cells), aliquots of 3 ml of cells were subjected to heat shock treatment at 42℃ for 1 h, followed by 2 h recovery at 37℃. The cells were allowed for incubation at 37℃ for 24 h. The cells were then analyzed for apoptosis by annexin V and PI staining using flow cytometer- (A) unstained control, (B) 37◦C and (C) 42◦C heat shock treated.

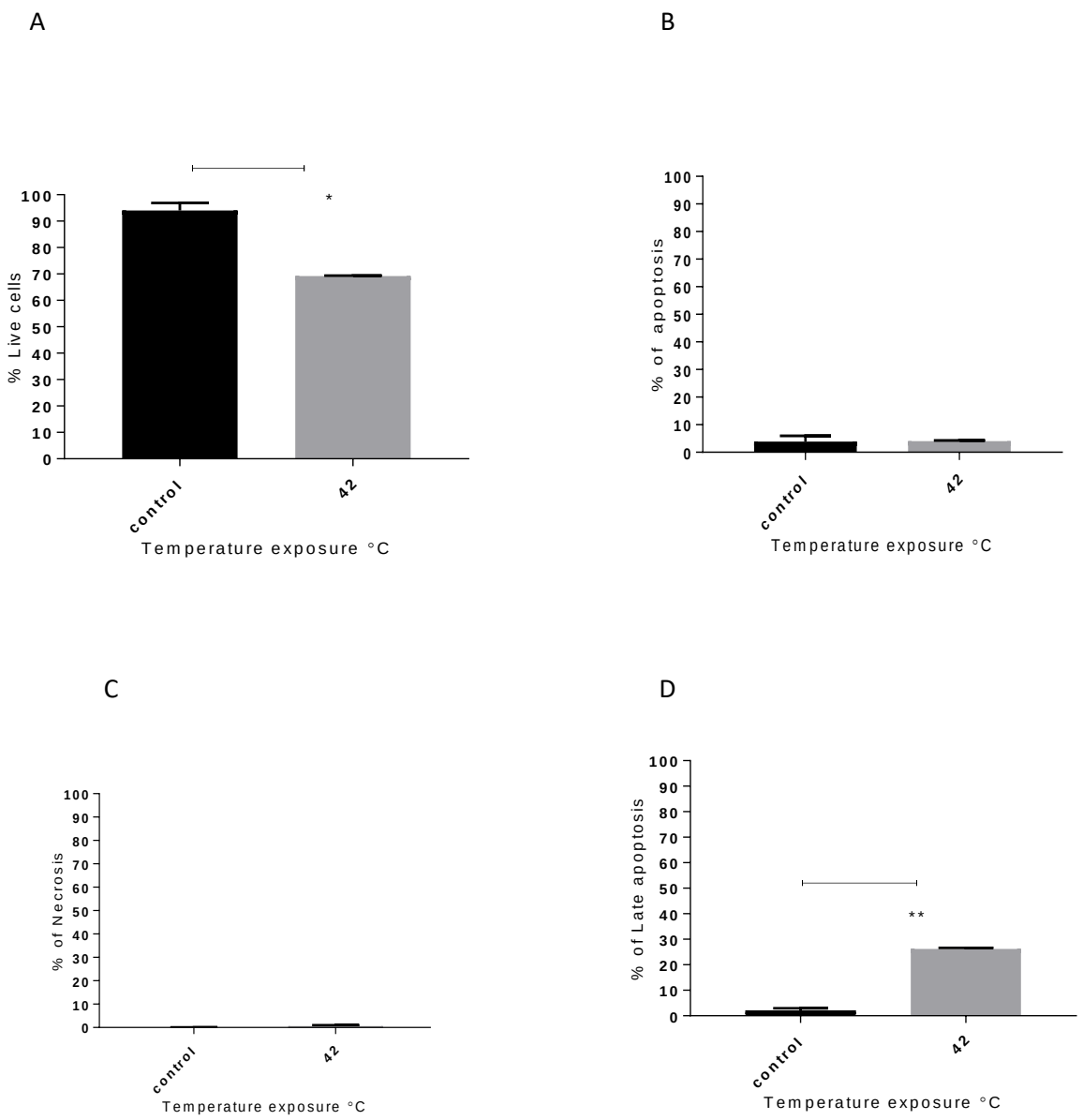


Fig: 4.3.23 Graphical determination of apoptosis by annexin V and PI staining in HT-29 cell lines exposed to 42 ℃. HT-29 cell lines at cell density (1 × 10^6 cells), aliquots of 3 ml of cells were subjected to heat shock treatment at 42◦C for 1 h, followed by 2 h recovery at 37℃. The cells were allowed for incubation at 37℃ for 24 h. The cells were then analyzed for apoptosis by annexin V and PI staining using flow cytometer. Statistical analysis performed using paired-t test represented mean±SD(P<0.05) n=3; 37 vs 42 (A) (P-0.0173**; (B) P-0.0914 insignificant; (C) P-0.0219*;(D) p-0.0085**).

4.4. Discussion

The aim of the work in this Chapter was to evaluate whether inhibiting HSPA1A protein activity using the inhibitors pifithrin - μ & pifithrin chloride resulted in increased effectiveness of UV exposure or hyperthermia in inducing cell death.

Firstly, the study evaluated the cytotoxicity induced by UV radiation. UV induced cell death (Fig (Fig 4.3.1,4.3.2 A, Table 4.1) as hypothesized, however no necrosis was observed by PI (Fig 4.3.2 B, Table 4.1). Results from the experiments suggests cell cytotoxicity by UV maybe due to apoptosis (Fig 4.3.2). Studies relates apoptotic signaling pathways including members of the caspase family - 3, 8 and 9, evidence to play a major role in stimulating UV assisted apoptosis in Leukaemia cancers (Salucci et al. 2013). Secondly, considering the defensive role of HSPA1A proteins (Mansilla et al. 2014), which encounter for the resistance of cancer cells to conventional treatments, studies have evidenced the potential role of pifithrin - μ in inhibiting HSPA1A proteins, which relates to enhanced sensitivity to treatments in several malignancies (Kaiser et al. 2011). The HSPA1A protein inhibitor compounds - Phenylethylsulphomide (Pifithrin - μ) and 2-(3-chlorophenyl) ethynesulfonamide (Pifithrin chloride) function in hampering the lysosome enzyme catalysed mechanism, which plays a major role in discarding of unwanted metabolic residues within cancer cells thus allowing successful proliferation (Mizushima et al. 2008; Jones et al. 2016).

Administration of HSPA1A protein inhibitors pifithrin - μ and pifithrin chloride on U937 cell lines displayed decline in cell viability with increased necrosis (Fig 4.3.3&4 A, Table 4.2) at 24 h and the necrosis declined upon prolonged exposure for 48 h (Fig 4.3.6 B Table 4.3), this may be due to disintegration of DNA due to prolonged treatment duration, which may have resulted in improper binding of the propidium iodide dye. Cytotoxic assessment with pifithrin chloride, which also expressed significant drop cell viability on U937 cell lines (Fig 4.3.7 A, Table 4.4) with enhanced necrotic effect at 24 h treatments (Fig 4.3.7 B, Table 4). Pifithrin chloride also showed a decline in necrosis similar to that of pifithrin - μ upon prolonged exposure for 48 h (Fig 4.3.8 B, Table 5).

Pifithrin - μ was selected for the combination experiments with UV as pifithrin - μ was found to be less cytotoxic than pifithrin chloride, and the study designed at experimenting whether

lower dose of pifithrin - μ could accelerate the rate of cytotoxicity with limited UV exposure. The two - pronged approach, ablation of HSPA1A protein activity using pifithrin - μ (20μM) and UV for 2 and 4 s (Fig 4.3.10, Table 4.6) failed to identify any significant variation as pifithrin -μ at this tested concentration itself produced considerable cytotoxicity. The results from the study interpreted enhanced cytotoxic response of UV irradiation with pifithrin - μ at low concentration (10μM) (Fig 4.3.11, Table 4.7) accelerated the rate of cytotoxicity in U937 cancer cell lines.

And these experimental results match the results of previous studies which presented enhanced cytotoxicity upon introducing radiation with other pifithrin forms α and β (Walton et al. 2005). Hence this combination treatment displayed the potential role of pifithrin - μ in accelerating apoptosis in leukaemia cancer cell line U937 and reduces the risk of prolonged radiation exposure, enhancing the therapeutic index of radiation treatment.

Having postulated the eminent role of HSPA1A proteins in shielding intracellular proteins from elevated heat, exposure to chemical toxins, ultra violet radiation cancer survival and resistance towards cancer treatments. The study also attributed to the positive response of HSPA1A proteins on hyperthermia treatment.

Intracellular heat shock proteins reaching the exterior, elicit signals to trigger the immune system identify the threat and respond accordingly. Previous report evidence differentiation of U937 cell lines upon mid hyperthermia exposure (Goliaei. 1998).

Recent findings reveal that, the transcription of these genes can be achieved indirectly by attenuating the intracellular link mediated through several other operons such as NEF- Nucleotide exchange factors and TPR - Tetratricopeptide chains, which works in close connection with HSPA1A protein resulting insensitiveness to natural host defense, promoting cancer survival (Assimon et al. 2013; Daming. 2016). The over expression of HSPA1A genes are studied to be key factors associated to cellular proliferation in most of the malignancies. Hence an explicit overview of function of these proteins at intracellular and extracellular levels may help improve conventional therapeutic index of different malignancies. (Multhoff. 2006; Daming. 2016).

Hyperthermia at 42℃ in U937 cell lines presented significant drop in cell viability (P 0.0025**) (Fig 4.3.12 A, Table 4.8), which was confirmed to be resulted from apoptotic cell death by annexin v and PI staining (Fig 4.3.13), which showed similar drop in viability of around 38% (Fig 4.3.14). Hyperthermia treatment encountered for 20% increase (Fig 4.3.15) in release of HSPA1A proteins compared to untreated cells maintained at 37℃ . Previous *in vitro* report exhibited significant release of HSPA1A in response to hyperthermia treatment, directing apoptosis in hepato - carcinoma cancers (Yang et al. 2016). Therefore, therapeutic modalities targeting HSPA1A may help in improving cancer treatments according to (Schildkopf et al. 2011).

This study also examined significant cytotoxic effects displayed by pifithrin - μ in colorectal cancer cell line HT-29 at varying concentrations for 24 h (Fig 4.3.16, Table 4.9) and 48 h (Fig 4.3.17, Table 4.10). Similar cytotoxic studies were studied using pifithrin chloride, which was found to be significantly more necrotic (Fig 4.3.18, Table 4.11) compared to pifithrin - μ at 24 h treatment exposure. Pifithrin chloride also presented similar reduction in necrosis as that of leukaemia cancer cell line U937 with 48 h prolonged exposure treatment (Fig 4.3.19, Table 4.12). This study further reveals the active participation of pifithrin - μ at low doses 5 and 10μM in accelerating the effect of UV radiation treatment (Fig 4.3.20, Table 4.13).

Hyper thermic treatment was studied to be effective as hypothesized against colorectal cancer survival at 42℃ (P 0.0349*, Fig 4.3.21), which were confirmed to be due to apoptotic cell death (Fig 4.3.21 & 4.3.22).

This chapter concludes that UV radiation, pifithrin - μ, pifithrin chloride and hyperthermia treatments are effective anti - cancer agents in both Leukemia cell line U937 and colorectal cancer cell line HT-29 *in vitro*. And pifithrin - μ at doses as low as 5 and 10μM combined with limited UV exposure duration of 2 and 4 s evidenced pronounced cytotoxicity compared to UV exposures at 2 and 4s independently in both the tested cancer cell lines.

Chapter 5

Intracellular delivery systems of chitosan and BSA

5.1 Introduction

The efficacy and efficiency of therapeutics relies on several factors such as prolonged bioavailability, targeted delivery and protection from enzymatic degradation. Direct administration of drugs often fails to produce expected results, due to poor absorption and interference of intestinal enzymes with the therapeutics resulting in inadequate drug bioavailability. Intriguingly, the exoskeleton of crustaceans possesses a component known as chitin, which is decalcified and deacetylated to extract chitosan which is an efficient natural polymeric carrier system which evidence to be successful for safe and targeted delivery of biological entities and chemotherapeutics (Saranya et al. 2011; Ghasemi et al. 2015).

Moreover, its high degradability and biocompatible nature makes chitosan nano drug combinations perfect candidate for drug delivering applications (Kalpana. 2010). The pH sensitive and mucus - adhesive characteristic feature of chitosan confines it not only to cancer drug deliveries but also for clinical conditions, in which drugs are required to be transported across the mucosal barriers (Artursson. 1994). Studies have presented the accelerated drug delivering ability of chitosan based microparticles and nanoparticles (Chua et al. 2012; Hou et al. 2014). These particles not only exhibited enhanced drug entrapment but also experienced prolonged drug activity *in vivo,* thus increasing the therapeutic potential of commercial drugs including atropine sulphate and folic acid (Addo et al. 2015; Driton et al. 2013). Reports show that proteins such as insulin embedded in chitosan polymer matrix were shown to promote greater drug retention and controlled release for prolonged time duration (Zhang. 2010; Nam et al. 2010).

Although the stability and bioavailability of drugs are well maintained within chitosan microgel (Marchand et al. 2009; Depani et al. 2013). The side effects produced by these systems remain unclear and still under exploration. Furthermore, studies have showcased the effective release and drug entrapment of glycerol phosphate cross linked chitosan microgels with the aid of dialysis bags, however the efficiency and cytotoxicity of β - glycerol phosphate

grafted microgel systems in human colon cancer cell lines has not been yet reported to our knowledge (Haddad et al. 2013).

In addition to chitosan, bovine serum albumin (BSA) formulated micro and nano based systems are shown to be excellent natural entities for several drug delivery applications (Yu et al. 2014; Casa et al. 2015; Antonio et al. 2016). Evidence suggests that BSA nano formulations can improve the therapeutic potential of chemotherapeutics including doxorubicin, paclitaxel, anthracycline etc. (Sahaet al. 2016). This chapter will investigate the effect of chitosan or BSA particles encapsulated with 5 - fluorouracil for an accelerated cytotoxicity on HT-29 colon cancer cell lines, such that, these particles if presented an enhanced drug cytotoxicity and found to be non - cytotoxic in drug free states, may be postulated safe and could be used for plasmid DNA transfection studies.

5.1.1 Aims and Hypotheses

To evaluate the application of intracellular drug delivery particles of chitosan or BSA proteins The objectives of the study are-

1.To evaluate the cytotoxicity of cross linking agent β - glycerol phosphate or glutaraldehyde in chitosan microgels or microparticles independently or in combination with 5 - fluorouracil on HT-29 colorectal cancer cell lines.

Hypotheses-

H0 chitosan microgels cross linked with β - glycerol phosphate or glutaraldehyde will not inhibit the growth of HT-29 cells in drug free state.

H1 chitosan microgels cross linked with β - glycerol phosphate or glutaraldehyde may inhibit the growth of HT-29 cells in drug free state.

2.To evaluate the effects of BSA microparticles independently or in combination with 5 - fluorouracil on HT-29 cancer cell lines.

Hypotheses-

H0 BSA microparticles will not induce growth inhibition of HT-29 cell lines in drug free form and hence may be safe intracellular delivery system.

H1 BSA microparticles may induce cell cytotoxicity on HT-29 cells in its intact form.

5.2 Methods

5.2.1 Cell culture and experimental design

Cells were cultured and maintained as illustrated in (section 2.3.1.2). Colorectal cancer cell lines HT-29 at 5×10^5 cells/ml seeding density were used for all the experiments.

5.2.2 Chitosan - β - glycerol phosphate microgels treatment

HT-29 cell lines were subjected to treatment with the chitosan microgels prepared as described in (section 2.3.8) with chemotherapeutic 5 - fluorouracil and drug free controls in EMEM cell culture medium. Drug free microgels, 5 - fluorouracil treated, and untreated cell lines were placed as controls. Microgel treatments were allowed for 24 h at 37℃ in incubator.

5.2.3 Chitosan - glutaraldehyde microparticle treatment

HT-29 cell lines were subjected to treatment with glutaraldehyde cross linked chitosan microparticles (prepared as illustrated in section 2.3.9) in EMEM cell culture medium.

5.2.4 Treatment with BSA microparticles

Microparticles of BSA was prepared using heat denaturation method as described in (section 2.3.10). HT-29 cell lines were treated with microparticles encapsulated with 5 - fluorouracil. Drug free microparticles, 5 - fluorouracil treated, and untreated cell lines were placed as controls. Microparticle treatment was allowed for 24 h at 37℃ in incubator.

5.2.5 Determination of cytotoxicity and microscopic observations

Cell cytotoxicity were assessed by MTS cell viability assay (section 2.3.4) following 24 h microgel/microparticle formulation treatments on HT-29 cell lines. The formulations thus prepared using chitosan or BSA were observed using scanning electron microscopy for the formation of microgels and microparticles.

5.3 Results

5.3.1 Microscopic observation of Chitosan β - glycerol phosphate microgels and chitosan microparticles

Chitosan β - glycerol phosphate crosslinked microgels prepared were observed for structural morphology determination by scanning electron microscopy (Fig 5.3.1 A, B, C) prior to *in vitro* cytotoxicity assays in colorectal cancer cell line HT-29. The microgels appeared to have densely scaffolded parallel structures at 1 KX, a magnified image revealed at 2 KX showcased tightly packed microspheres in appearance and the microsperes were visible upto a magnification to 10.13 KX.

The chitosan - glutaraldehyde microparticles under scanning electron microscopic observation produced image of microspheres with a ring - shaped appearance at magnification of 1.44 K X, 10µM scale (Fig 5.3.2).

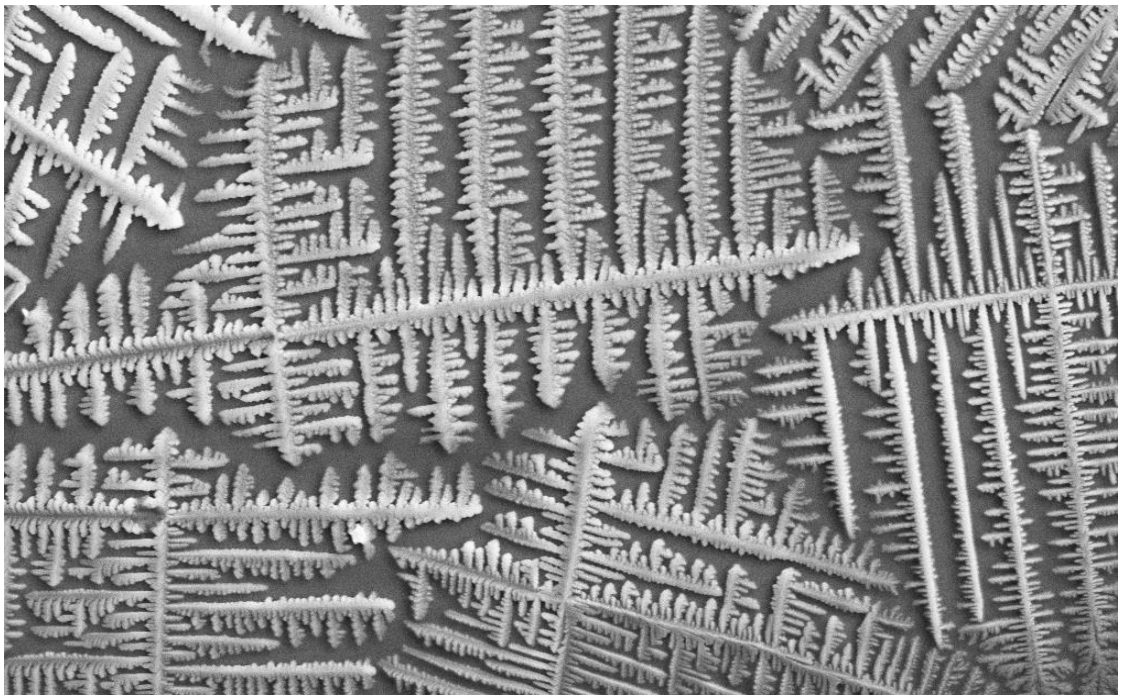


Fig: 5.3.1A Scanning electron microscopic observation of β - glycerol phosphate crosslinked chitosan microgels at magnification of(A) 1.00 K X,10µm scale.

Fig: 5.3.1 B Scanning electron microscopic observation of β - glycerol phosphate crosslinked chitosan microgels at magnification of (B)2.00 K X, 10µm scale.

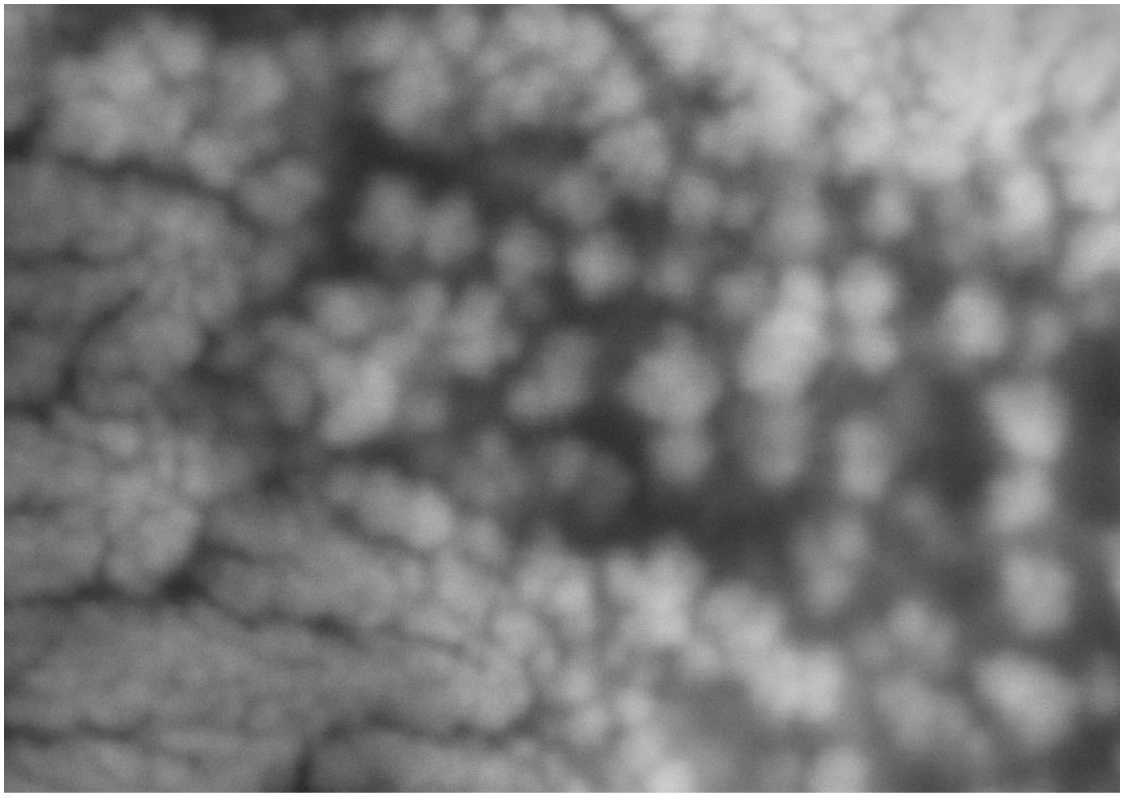


Fig: 5.3.1 C Scanning electron microscopic observation of β - glycerol phosphate crosslinked chitosan microgels at magnification of (C) 10.13 K X, 2µm scale.

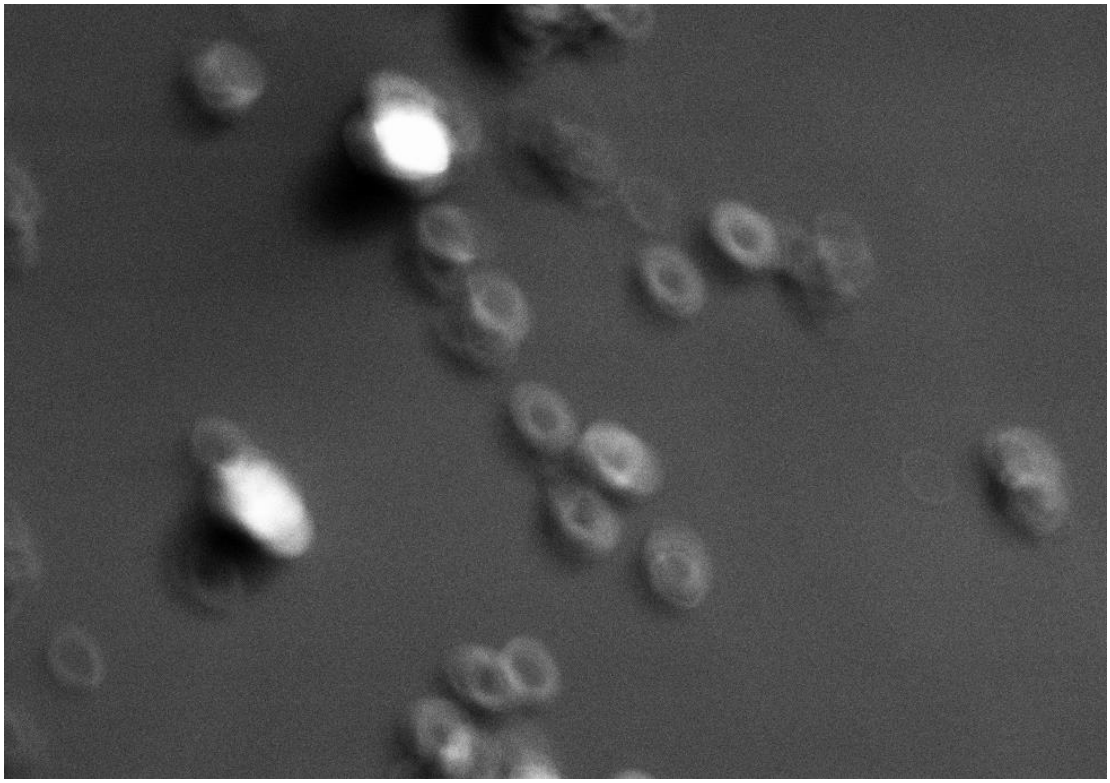


Fig: 5.3.2 Scanning electron microscopic observation of glutaraldehyde crosslinked chitosan microparticles at magnification of 1.44 K X,10µm scale.

5.3.2 Cytotoxicity of β - glycerol phosphate on HT-29 cell

The following experiment were performed to determine the cytotoxicity of cross-linking agent β - glycerol phosphate in chitosan microgels on colorectal cancer cell lines HT-29. The microgels prepared (section 2.3.8) were then diluted using EMEM medium containing 10% FBS and added to HT-29 cell lines pre-seeded in 96 well assay plates. 5 - fluorouracil free microgels, 5 - fluorouracil treated, untreated cells and dead cells were placed as controls. The cells were then allowed for incubation for 24 h at 37℃ in incubator.

The MTS assay presented decreased cell viability on HT-29 cancer cell lines with microgels prepared with all the tested concentrations of β - glycerol phosphate content, with significant difference of ($P<0.0001$) with reference to untreated controls (Fig 5.3.3). The results demonstrated no significant difference between cytotoxicity induced by tested concentrations 1 and 5% β - glycerol phosphate microgels, however the cytotoxicity exerted by higher concentrations 5 and 10% were significant as presented in Table 5.1.

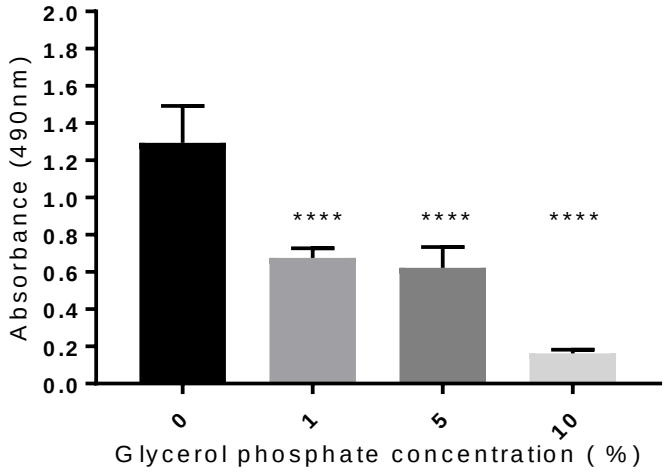


Fig: 5.3.3. Effect on cell viability by β - glycerol phosphate concentration in chitosan microgels on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5×10^5 cells/ml) were used in all experiments. Cells were then treated with chitosan microgels of varying concentrations of glycerol phosphate. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one- way ANOVA, with post hoc Turkey's multiple comparisons test. Significant differences between glycerol phosphate concentrations with reference to the untreated control are indicated by * ($p<0.05$) mean±SD; n=4.

Table 5.1 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay

Beta -glycerol phosphate (%)	Significance	P value
0 vs 1	****	P<0.0001
0 vs 5	****	P<0.0001
0 vs 10	****	P<0.0001
1 vs 5	ns	P 0.9168
5 vs 10	***	P 0.0006

5.3.3 Effect of 5 - fluorouracil encapsulated chitosan microgels on HT-29 cell lines

Colorectal cancer cell lines HT-29 were subjected to treatment with chitosan microgels of varying β - glycerol concentration and encapsulated with 5 - fluorouracil at concentrations 5 and 10 µM for 24 h incubation at 37℃.

Cells treated with 5 - fluorouracil, drug free microgels and untreated cell lines were plated as controls. The reduced cell viability exerted by drug free microgels of varying concentrations of β - glycerol phosphate concentrations tested were consistent with previous experiments (Fig 5.3.3).

The chitosan microgels cross linked with 10% of β - glycerol phosphate grafted with 5 - fluorouracil expressed significant drop in cell viability ($P<0.0001$) in contrast to 5 - fluorouracil in its intact form at both tested concentrations (Fig 5.3.4, Table 5.2). Upon reducing the ratio of β - glycerol phosphate in the microgels to 0.5% w/v (Fig 5.3.7 Table 5.5) the drug free microgel showed significant decline in cell viability with enhanced effect with the 5 - fluorouracil combination on HT-29 cell lines. Even though chitosan microgels with β - glycerol phosphate of 0.5% w/v (Fig 5.3.7, Table 5.5) expressed considerably less cytotoxicity in its intact form compared to toxicity exerted by higher β - glycerol phosphate concentrations from previous experiments, the 5 - fluorouracil combinations of 0.5% w/v β - glycerol phosphate did not produce expected reduction in cell viability of HT-29 cell lines as compared to combinations tested with higher β - glycerol phosphate content.

5.3.4 Effect of 5 - fluorouracil encapsulated glutaraldehyde linked chitosan microparticles on HT-29 cell lines

Colorectal cancer cell lines HT-29 were subjected to treatment with chitosan microparticles encapsulated with 5 - fluorouracil at concentrations 5 and 10 µM for 24 h incubation at 37℃. Cells treated with 5 - fluorouracil independently, drug free microparticles and untreated cell lines were placed as controls. The chitosan microparticles cross linked with glutaraldehyde and combined with 5 - fluorouracil (section 2.3.9) and drug free microparticles expressed significant drop in cell viability ($P<0.0001$) in contrast to 5 - fluorouracil in its intact in both the tested concentrations and the untreated controls (Fig 5.3.8 Table 5.6).

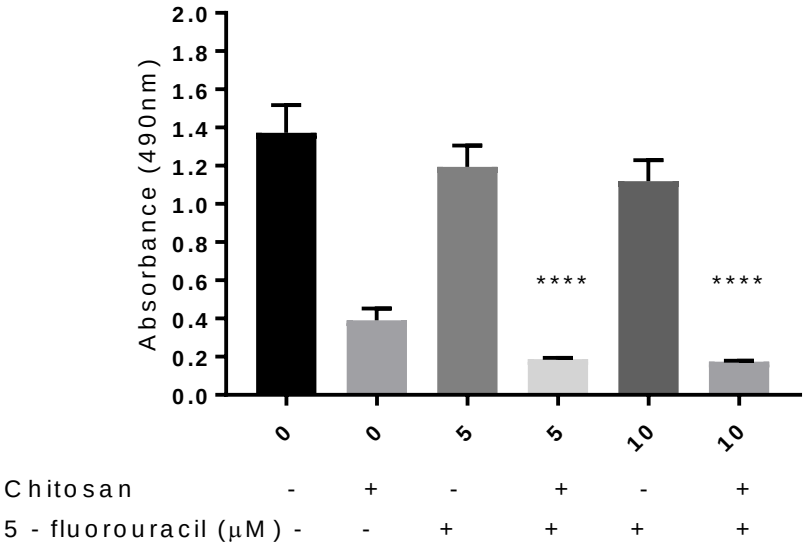


Fig: 5.3.4 Effect on cell viability by chitosan - β - glycerol phosphate (10 %) – 5 - fluorouracil microgels on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5×10^5 cells/ml) were used in all experiments. Cells were then treated with chitosan microgel (glycerol phosphate- 10 %) with 5 - fluorouracil 5 & 10μM and allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one- way ANOVA, with post hoc Turkey's multiple comparisons test. Significant differences between 5 - fluorouracil and chitosan-5 - fluorouracil at concentrations 5 and 10 μM are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 5.2 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - Fluorouracil (μM)	**Significance**	**P value**
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-5	ns	P 0.1101
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-10	*	P 0.0102
Chitosan (-) 5 - fluorouracil (-) vs Chitosan (+)	****	P<0.0001
5 - flurouracil-5 vs Chitosan (+) 5 - fluorouracil -5 (+)	****	P<0.0001
5 - flurouracil-10 vs Chitosan (+) 5 - fluorouracil -10 (+)	****	P<0.0001

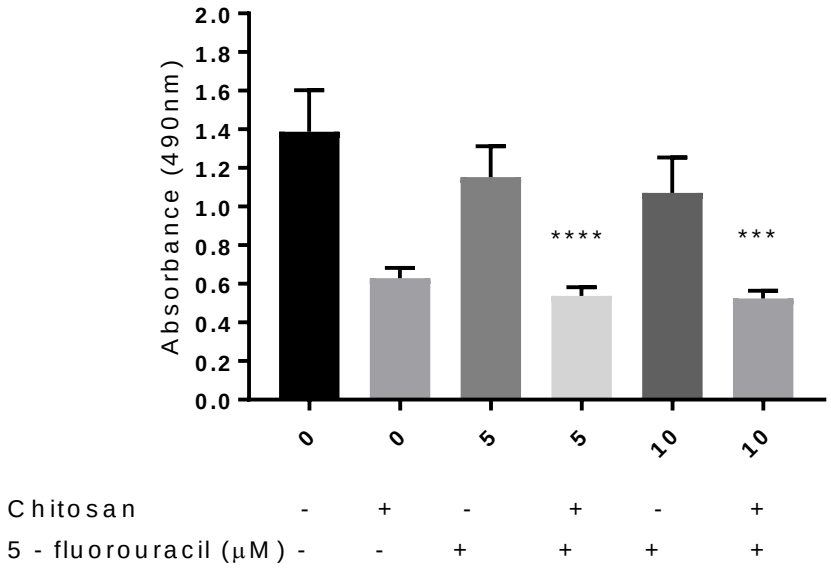


Fig: 5.3.5 Effect on cell viability by chitosan - β - glycerol phosphate (5 %) - 5 - fluorouracil microgels on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5×10^5 cells/ml) were used in all experiments. Cells were then treated with chitosan - 5 - fluorouracil microgels of 5 & 10μM. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Turkey's multiple comparisons test. Significant differences between 5 fluorouracil and chitosan -5 - fluorouracil combinations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 5.3 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - fluorouracil (μM)	**Significance**	**P value**
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-5	ns	P 0.1764
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-10	ns	P 0.0581
Chitosan (-) 5 - fluorouracil (-) vs Chitosan (+)	****	P<0.0001
5 flurouracil-5 - vs Chitosan (+) 5 - fluorouracil -5 (+)	****	P<0.0001
5 - flurouracil-10 vs Chitosan (+) 5 - fluorouracil -10 (+)	***	P 0.0006

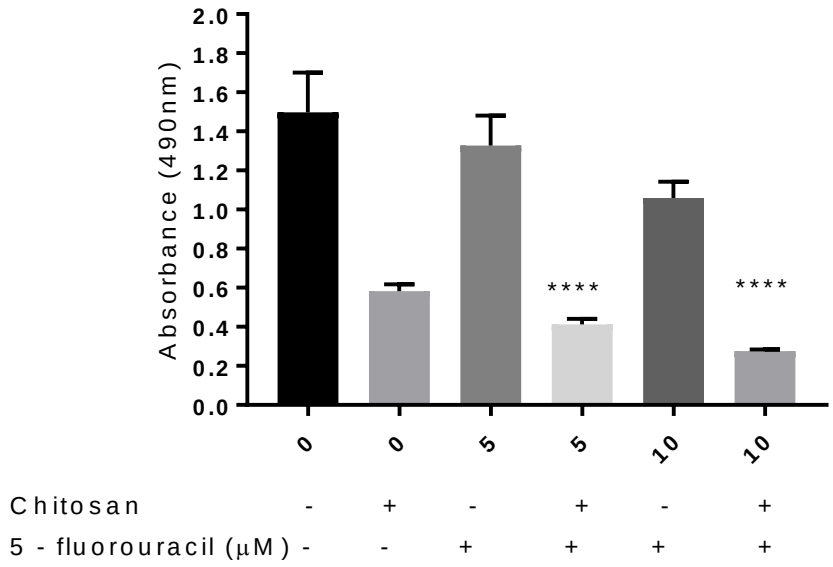


Fig: 5.3.6 Effect on cell viability by chitosan - β - glycerol phosphate (1 %) - 5 - fluorouracil microgels on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5 × 10^5 cells/ml) were used in all experiments. Cells were treated with chitosan- 5 - fluorouracil 5 & 10μM microgels. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Sidaks multiple comparisons test. Significant differences between 5 - fluorouracil vs chitosan - 5 - fluorouracil combination is indicated by **** ($p<0.05$) mean±SD; n=4.

Table 5.4 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - Fluorouracil (μM)	**Significance**	**P value**
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-5	ns	P 0.3039
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-10	***	P 0.0003
Chitosan (-) 5 - fluorouracil (-) vs Chitosan (+)	****	P<0.0001
5 - flurouracil-5 vs Chitosan (+) 5 - fluorouracil -5 (+)	****	P<0.0001
5 - flurouracil-10 vs Chitosan (+) 5 - fluorouracil -10 (+)	****	P<0.0001

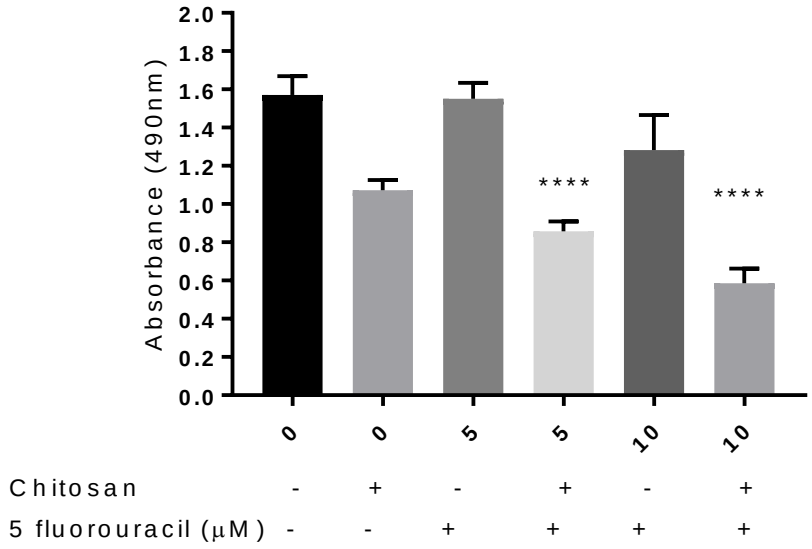


Fig: 5.3.7 Effect on cell viability by chitosan - β - glycerol phosphate (0.5 %) - 5 - fluorouracil microgels on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5×10^5 cells/ml) were used in all experiments. Cells were then treated with 5 - fluorouracil and chitosan - microgels of 5 - fluorouracil at 5 & 10 μM. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Turkey's multiple comparisons test. Significant differences between 5 - fluorouracil vs chitosan - 5 - fluorouracil combinations are indicated by **** ($p<0.05$) mean±SD; n=4.

Table 5.5 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - fluorouracil (μM)	**Significance**	**P value**
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-5	ns	P 0.9998
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-10	*	P 0.0102
Chitosan (-) 5 - fluorouracil (-) vs Chitosan (+)	****	P<0.0001
5 - flurouracil-5 vs Chitosan (+) 5 - fluorouracil -5 (+)	****	P<0.0001
5 - flurouracil-10 vs Chitosan (+) 5 - fluorouracil -10 (+)	****	P<0.0001

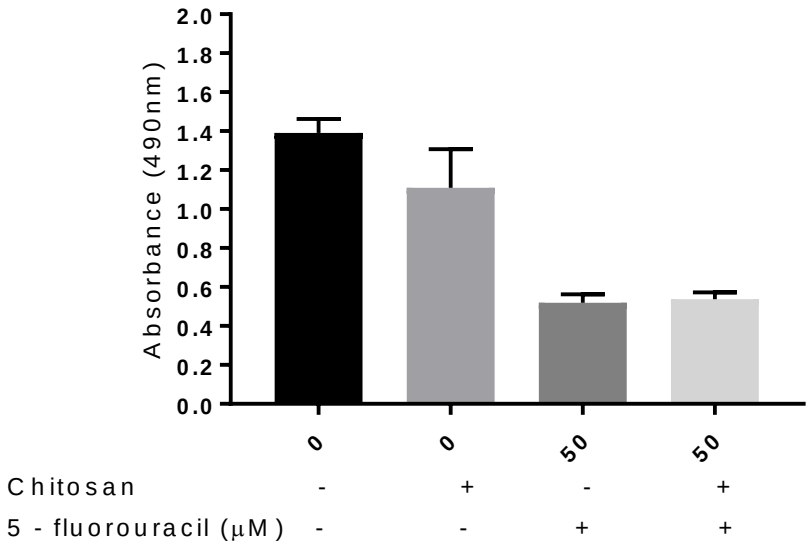


Fig: 5.3.8 Effect of cell viability by 5 - fluorouracil (glutaraldehyde cross linked) on HT-29 cell lines. Adherent cells of HT-29 seeded at cell density (5 × 10^5 cells/ml) were used in all experiments. Cells were then treated with microparticles of Chitosan - 5 - fluorouracil 50 μM, along with respective controls. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Turkey's multiple comparisons test. The analysis depicted insignificant differences between chitosan -5 - fluorouracil and 5 fluorouracil (50 μM) are indicated by ($p<0.05$) mean±SD; n=4.

Table 5.6 Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - fluorouracil (μM)	**Significance**	**P value**
Chitosan (-) 5 - fluorouracil (-) vs Chitosan (+)	*	P 0.0130
Chitosan (-) 5 - fluorouracil (-) vs 5 - flurouracil-50	****	P<0.0001
Chitosan (+) vs chitosan (+)-5 - flurouracil-50	****	P<0.0001
Chitosan (+) vs chitosan (+)-5 - flurouracil-50	****	P<0.0001
5 - fluorouracil (+) vs chitosan (+)-5 - flurouracil-50	ns	P 0.9924

5.3.5 Effect of BSA microparticles encapsulated with 5 - fluorouracil on HT- 29 cell lines

The following experiments were performed to determine the effect of BSA microparticles encapsulated with 5 - fluorouracil on HT- 29 cell lines. The cell lines were treated with BSA microparticles combined with 5 - fluorouracil. The cells were then incubated for 24 h at 37℃ in incubator. Cells were treated with 5 - fluorouracil independently, drug free microparticles and untreated cell lines were placed as controls. The microparticles size ranged between 1.9 - 3.8µM in size according to scanning electron microscopy (Fig 5.3.9). MTS cell viability assay expressed cytotoxicity with drug free microparticles which were insignificantly variant with 5 - fluorouracil encapsulated particle treatments on HT-29 cell lines (Fig 5.3.10, Table 5.7).

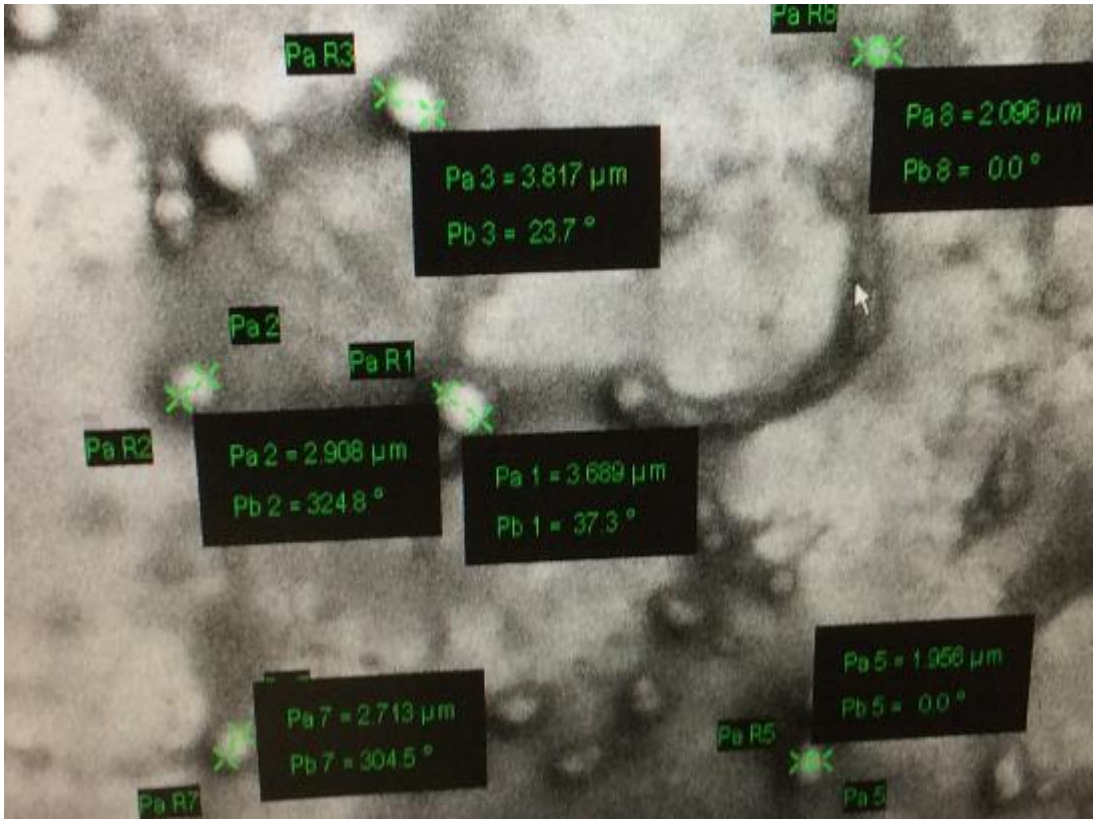


Fig: 5.3.9 Scanning electron microscopic observation of BSA protein microparticles at magnification of (A) 1.44 K X,10µM scale.

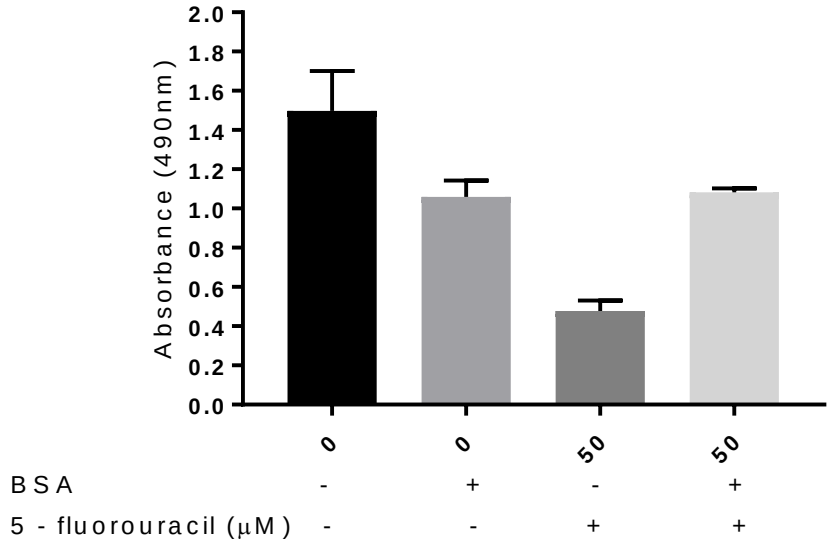


Fig: 5.3.10 Effect of cell viability by BSA microparticles loaded 5 - fluorouracil on HT-29 cell lines. HT-29 cell lines seeded onto 96 well assay plates at cell density (5×10^5 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with microparticles of BSA - 5 - fluorouracil at 50μM. The cells were allowed for 24 h incubation at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Turkeys multiple comparisons test. Significant differences between BSA vs BSA – 5 - fluorouracil concentrations are indicated by ($p<0.05$) mean±SD; n=4.

Table 5.7Statistic presentation of Turkey's multiple comparisons test (ns* insignificant)

MTS Assay		
5 - fluorouracil (μM)	**Significance**	**P value**
BSA (-) 5 - fluorouracil (-) vs 5 - flurouracil-50	****	P<0.0001
BSA (-) 5 - fluorouracil (-) vs BSA (+)	***	P 0.0007
BSA (-) 5 - fluorouracil (-) vs BSA (+)-5 - flurouracil-50	**	P 0.0012
5 - flurouracil-50 vs BSA-5 - flurouracil-50	****	P<0.0001
BSA (+) – 5 - fluorouracil (-) vs BSA (+) – 5 - fluorouracil (+)	ns	P 0.990

5.4 Discussion

The objective of the second phase of the study was to evaluate the cytotoxicity of chitosan or BSA microparticle/microgel drug delivery systems on colorectal cancer cell line HT-29. The chitosan microgels tested not only enhanced cytotoxic activity on HT-29 cell lines in drug frees states itself, but also failed to produce expected drug cytotoxicity with low concentrations of the cross - linking agent used to graft the microgels. The microparticles of BSA and chitosan were also found to be toxic to HT-29 cell lines in its drug free form.

Studies suggests the potential application of chitosan modified microgel systems for drug encapsulation and release (Mahattanadul et al. 2016; Puga et al. 2013). Although previous report showed, chitosan β - glycerol phosphate microgels as efficient in drug encapsulation and release, these microgels in its free state are prone to be cytotoxic in a concentration - based manner in fibroblast cells in mouse (Depani et al. 2013). The reports from this present study also reveal the toxicity exerted by chitosan - β - glycerol phosphate microgels in colorectal cancer cell line HT-29.

The cytotoxicity of the microgels were studied to be resulted from the toxicity of the cross-linking agent β - glycerol phosphate in a dose dependent manner (Fig 5.3.3, Table 5.1). The chitosan microgels cross linked with all the different concentration of β - glycerol phosphate produced accelerated cytotoxicity with 5 - fluorouracil encapsulations in all concentration compared to 5 - fluorouracil in its native form (Fig 5.3.4 – 5.3.7).

Although microgels modified with the lowest β - glycerol phosphate concentration tested 0.5% expressed considerably less toxicity in drug free state compared to its higher doses in HT-29 cell lines, 5 - fluorouracil encapsulation displayed slight decline in cytotoxicity compared to 5 - fluorouracil - chitosan microgels with higher β - glycerol phosphate concentrations tested, this may be due to the lower quantity of β - glycerol phosphate (0.5%), which maybe insufficient enough to trap the all the 5 - fluorouracil incorporated. Apart from chitosan microgels, the glutaraldehyde cross linked chitosan microgels also presented cytotoxicity in drug free state (Fig 5.3.8).

Besides the application of chitosan, BSA protein - based nanoparticles are proved to be efficient and safe intracellular drug transport systems, expressing enhanced therapeutic

effect (Yu et al. 2014). However, in this present study, although the BSA microparticles were successfully formed (Fig 5.3.9), the results showed no fluctuation in 5 - fluorouracil encapsulations in contrast to drug in its native state (Fig:5.3.10), this may be due to the large size of the particles thus formed which failed to entrap the 5 - fluorouracil used in the study.

Due to the cytotoxicity exerted by chitosan or BSA based delivery systems on the growth of HT-29 cells in their drug free forms, these particles were found unsuitable for use in transfection studies in the later part of the study. Hence, the thesis did not further investigate the encapsulation efficiency or drug release profiles of these delivery systems, as the use of these particles were found not suitable for intracellular delivery application in this study.

The scanning electron microscopic images of chitosan or BSA based delivery systems were observed to be morphologically similar to previous literatures (Depani et al. 2013; Yu et al. 2014). However, there are chances for artefact appearances in the images produced, which may be due to the physical or chemical alteration, resulted from dehydration process during sample fixation for scanning electron microscopic analysis.

Hence the findings in this chapter collectively stated that all the delivery systems tested were found inadequate due to the cytotoxicity exerted by subsequent cross-linking agents β - glycerol phosphate or glutaraldehyde in chitosan microgels or microparticles and BSA microparticles in drug free states on colorectal cancer cell line HT- 29.

Chapter 6

Effect of p21 plasmid cDNA transfection and HSPA1A inhibition in colorectal cancer cell line HT-29

6.1 Introduction

Cancer cells are developing resistance to conventional therapeutics (Axelrod et al. 2017), even with high dosage administration, hence the necessity of developing highly personalised treatment strategies is at the top priority. The p21 gene product functions as negative attenuator of DNA replication, in response to translation of several operons including PCNA, P53 genome, most importantly, these proteins are unique in their mechanism of interactive binding of cyclin dependent kinase inhibitors, attenuating cancer proliferation (Pavelic et al. 2008). Although, in some malignant cells, the function of p21 is resumed with respect to the malfunctional status of wild type p53 gene, in cancers possessing functional p53, p21 protein functions as potent negative regulator of cell cycle (Galanos et al. 2016).

Research, over the years has illustrated the eminent role of several genes, pre - programmed in cell cycle regulation in different cancer types, making explicit the vital role of tumour suppressor gene p21 in arresting cancer proliferation, suggesting its therapeutic potential in cancer gene therapy (Abbas et al. 2009; Pasz et al. 2001; Van Etten et al. 2002).

Eukaryotic cells under physiological stress response exhibits elevated levels of stress protein HSPA1A, which are studied to participate closely with signalling pathways assisting a protective role under cellular stress, enhancing tumorigenicity. According to recent reports, Inhibition of HSPA1A improved the sensitivity to chemotherapeutics in colorectal cancer (Goloudina et al. 2015; Jagadish et al. 2016). Recent studies reveal the resistance acquired by 5 - fluorouracil in colorectal cancer cells, due to the elevated levels of HSPA1A, expressing its defensive role against apoptosis (Grivicich et al. 2007).

However, the role of HSPA1A proteins in p21 tumor suppressor protein induced cancer arrest is not reported elsewhere according to our knowledge, and hence this present study

investigated a two - pronged approach inhibition of HSPA1A protein activity and simultaneous overexpression of suppressor protein p21.

In the previous experiments (chapter 5), it was determined that chitosan and BSA based intracellular delivery systems were not suitable for p21 - plasmid DNA delivery applications, so a different transfection approach using lipofectamine 2000 was performed.

Previous studies, evidence the insensitiveness of several chemotherapeutics is due to elevated levels of HSPA1A, expressing its defensive role against apoptosis in colorectal cancer cells (Grivicich et al. 2007). Hence, repression of HSPA1A proteins evidenced to improve the chemotherapeutic sensitivity in colorectal cancers (Goloudina et al; Sherman. 2015; Jagadish. et al. 2016). This work postulated the inhibition of HSPA1A in combination with increased p21 would enhance the apoptosis induced by p21 plasmid cDNA and may be a promising therapeutic strategy for the treatment of colorectal cancers.

6.1.1 Aims and hypotheses

The aims of this chapter were to evaluate-

1. The effect of overexpression of p21 protein through genetic approach in inducing apoptosis in colorectal cancer cell lines HT-29.

Hypotheses-

H0 transfection with p21 plasmid cDNA will not arrest the growth of HT-29 cells.

H1 p21 plasmid cDNA transfection may arrest the growth of HT-29 cells.

2. Whether inhibition of HSPA1A proteins and p21 overexpression would accelerate the apoptosis induced by p21 plasmid cDNA transfection in HT-29 cell lines.

Hypotheses-

H0 the inhibition of HSPA1A in combination with increased p21 will not accelerate apoptosis in HT-29 cells.

H1 the inhibition of HSPA1A in combination with increased p21 will enhance apoptosis in HT-29 cells.

6.2 Methods

6.2.1 Cell culture

Colorectal cancer cell lines HT-29, were cultured as described in (Secion 2.3.1). Cells at seeding density 5×10^5 cells/ml were used in all experiments.

6.2.2 p21 plasmid DNA isolation, extraction, purification, transformation and transfection

p21 plasmid cDNA was provided in *Escherichia coli* as stabbed agar cultures. Isolated bacterial colonies were produced by streaking the culture onto Lactose agar plates supplemented with ampicillin antibiotic, provided 37℃ in incubator for 24 h. Batch cultures were prepared in lactose broth medium Bacterial stock cultures were maintained in glycerol at -80℃ for future analysis. The plasmid cDNA was extracted from the bacterial cells using endo free maxi prep kit as illustrated in (section 2.3.13).

The extracted plasmid cDNA was quantified by DNA nano drop. The DNA was sequenced with aid from Euro fins laboratory services and confirmed for full length human P21 coding sequence. The sequence was further confirmed for p21 by blasting the sequence using NCBI nucleotide blast tool. The p21 plasmid cDNA was also analysed by agarose gel electrophoresis. The PmT5 vector was restricted using digestion enzymes Ndel and BamHI as illustrated in (section2.3.15), to remove the PMT5 backbone and religate it to generate a vector only control.

The restricted PmT5 vector DNA was separated by agarose gel electrophoresis, extracted from the gel fragment, repaired for blunt ends, ligated, purified and transformed into DH5α competent cells. The p21 plasmid cDNA which was transfected using lipofectamine 2000 in colorectal cancer cell line HT-29 according to manufacturer's protocol. The experimental steps from plasmid cDNA extraction to transfection were performed according to the respected commercial kit instructions provided.

6.2.3 Pifithrin - μ assisted inhibition of HSPA1A protein and p21 plasmid cDNA transfection

HT- 29 cells were seeded onto 96 well assay plates at a concentration of 5×10^5 cells/ml and allowed for incubation for 24 h. Following attachment, the cells were treated with pifithrin - μ (15μM) and p21 plasmid cDNA in lipofectamine 2000 in serum free EMEM cell culture

medium devoid of antibiotics. Untreated, PmT5 vector in lipofectamine, pifithrin - μ treated cell lines were placed as controls.

6.2.4 Measurement of cell viability, apoptosis, necrosis and protein expression profiles.

The subsequent treatments were allowed for 24 h and cells were analyzed for cytotoxicity by MTS assay as illustrated in (Section 2.3.4).

Flow cytometric analysis were also used to confirm the percentage of apoptosis and necrosis by annexin V and PI staining method as illustrated in (section 2.3.6)

6.2.5 Observation of apoptosis and protein expression profiles

HT-29 cells upon subsequent transfection treatments were observed for cytotoxicity under light microscopy (Fig 6.3.1). p21 plasmid cDNA and vector plasmid DNA transfected HT-29 cell lines were pooled and probed with FITC labelled p21 primary antibodies for protein expression profiles as illustrated in (section 2.3.7.4, 2.3.7.5) by flowcytometry. The transfected cells were confirmed for apoptosis using annexin V and PI staining as illustrated in (section 2.3.6) using flow cytometer.

6.2.6 Experimental design of p21 - plasmid cDNA - extraction, purification and transfection

Fig 6.2.6.1Isolated colonies of *Escherichia coli* containing p21 plasmid cDNA

⇩

Batch culturing of p21 plasmid cDNA inserted *Escherichia coli* in Luria-Bertani broth as illustrated in (section 2.3.12.1)

⇩

Plasmid cDNA extracted and purified by Qiagen Endofree maxi - plasmid prep protocol as illustrated in (section 2.3.13)

⇩

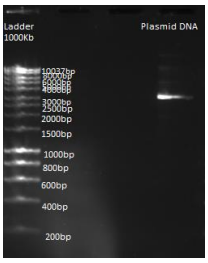


Fig 6.2.6.2 Identification of p21 plasmid DNA by agarose gel - electrophoresis

⇩

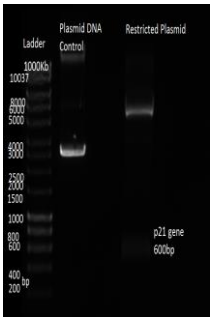


Fig 6.2.6.3 Restriction digest of p21 plasmid cDNA from vector DNA

⇩

DNA blunt end repair, purification of repaired DNA, ligation and sub cloning of vector DNA onto DH5 alpha *E. coli* cells (sections 2.3.19.1 – 2.3.19.3)

⇩

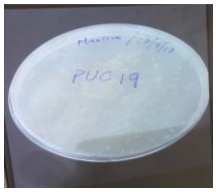


Fig 6.2.6.4 Transformation of PmT5 vector in DH5 alpha *E. coli* cell

6.2.7 Sequencing of p21 plasmid cDNA by Eurofins laboratories, UK

pBABE3 reverse sequencing

TCACAGGCATCTACTGAGTGGACCCAACGCATGAGAGGACAGTGCCAAGCAAGCAACTCA
AATGTCCCACCGGTTGGGCATGGCCAGGTAGCCTATGCTGTGTCTGGACGTCCTCCTGCT
GGTATAGTTATTTTAAAATCAGAAGGACAGGGAAGGGAGCAGTGGTTCACGCCTGTAATC
CCAGCAATTTGGGAGGCCAAGGTGGGTAGATCACCTGAGATTAGGAGTTGGAGACCAGCC
TGGCCAATATGGTGAAACCCCGTCTCTACCAAAAAAACAAAAATTAGCTGAGCCTGGTCA
TGCATGCCTGGAATCCCAACAACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCAGG
AGGCGGAGATTGCAGTGAGCCAAGATTGTGCCACTGCACTCCAGCTTGGTTCCCAATAGA
CCCCGCAGGCCCTACAGGTTGTCTTCCCAACTTGCCCCTTGCTCCATACCACCCCCCTCC
ACCCCATAATATTATAGAAGGACACCTAGTCAGACAAAATGATGCAACTTAATTTTATTA
GGACAAGGCTGGTGGGCACTGGAGTGGCAACTTCCAGGGCCAGGAGAGGCACTGGGGAGG
GGTCACAGGGATGCCACCCGGGATCCCCCGGGCTGCAGGAATTCGATATCTGCAGAATTC
GGCTTTAGAATTCTTAGGGCTTCCTCTTGGAGAAGATCAGCCGGCGTTTGGAGTGGTAGA
AATCTGTCATGCTGTCCTGCCGCCGTTTTCGACCCTGAGAGTCTCCAGGTCCACCTGGGG
ACCCTTCAGCCTGCTCCCCTGAGCGAGGCACAAGGGTACAAGACAGTGACAGGTCCACAT
GGTCTTCCTCTGCTGTCCCCTGCAGCAGAGCAGGTGAGGTGCCAGGCCGCCTGCCTCC

CMV forward sequencing

GCAGAGCTCGTTTAGTGAACCGTCAGAATTCAGATCTGGTACCACGCGTATCGATAAGCT
TGCATGCCTGCAGGTCGACTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC
ATGGACTACAAAGACGATGACGATAAAGCAAGAAGAGCATCTGTGCATATGTCAGAACCG
GCTGGGGATGTCCGTCAGAACCCATGCGGCAGCAAGGCCTGCCGCCGCCTCTTCGGCCCA
GTGGACAGCGAGCAGCTGAGCCGCGACTGTGATGCGCTAATGGCGGGCTGCATCCAGGAG
GCCCGTGAGCGATGGAACTTCGACTTTGTCACCGAGACACCACTGGAGGGTGACTTCGCC
TGGGAGCGTGTGCGGGGCCTTGGCCTGCCCAAGCTCTACCTTCCCACGGGGCCCCGGCGA
GGCCGGGATGAGTTGGGAGGAGGCAGGCGGCCTGGCACCTCACCTGCTCTGCTGCAGGGG
ACAGCAGAGGAAGACCATGTGGACCTGTCACTGTCTTGTACCCTTGTGCCTCGCTCAGGG
GAGCAGGCTGAAGGGTCCCCAGGTGGACCTGGAGACTCTCAGGGTCGAAAACGGCGGCAG
GACAGCATGACAGATTTCTACCACTCCAAACGCCGGCTGATCTTCTCCAAGAGGAAGCCC
TAAGAATTCTAAAGCCGAATTCTGCAGATATCGAATTCCTGCAGCCCGGGGGATCCCGGG
TGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTG
CCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTC
TATAATATTATGGGGTGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACC
TGTAGGG

6.3 Results

6.3.1 Effect of p21 plasmid cDNA transfection and pifithrin - μ mediated HSPA1A inhibition and p21 plasmid cDNA transfection in HT-29 colorectal cancer cell line

HT-29 cells transfected with the plasmid cDNA containing full length p21 were observed for morphology changes suggesting cell death using light microscope (Fig 6.3.1). The cells exhibited significant decrease in cell viability $P<0.0001$ by MTS cell viability assay with reference to the respected controls. (Fig 6.3.2). This decline in cell viability was confirmed by annexin V and PI staining assay by flow cytometer. Flow cytometer interpreted total apoptotic rate of 58.1 **%** (Fig 6.3.3 C) for the p21 transfected cell lines. pifithrin - μ assisted inhibition of HSPA1A protein activity with p21 plasmid DNA transfection presented a pronounced arrest of 90% of the treated population of HT-29 cells within 24 h (Fig 6.3.3E), which was approximately 62**%** higher than treatment with pifithrin - μ independently (Fig 6.3.3D).

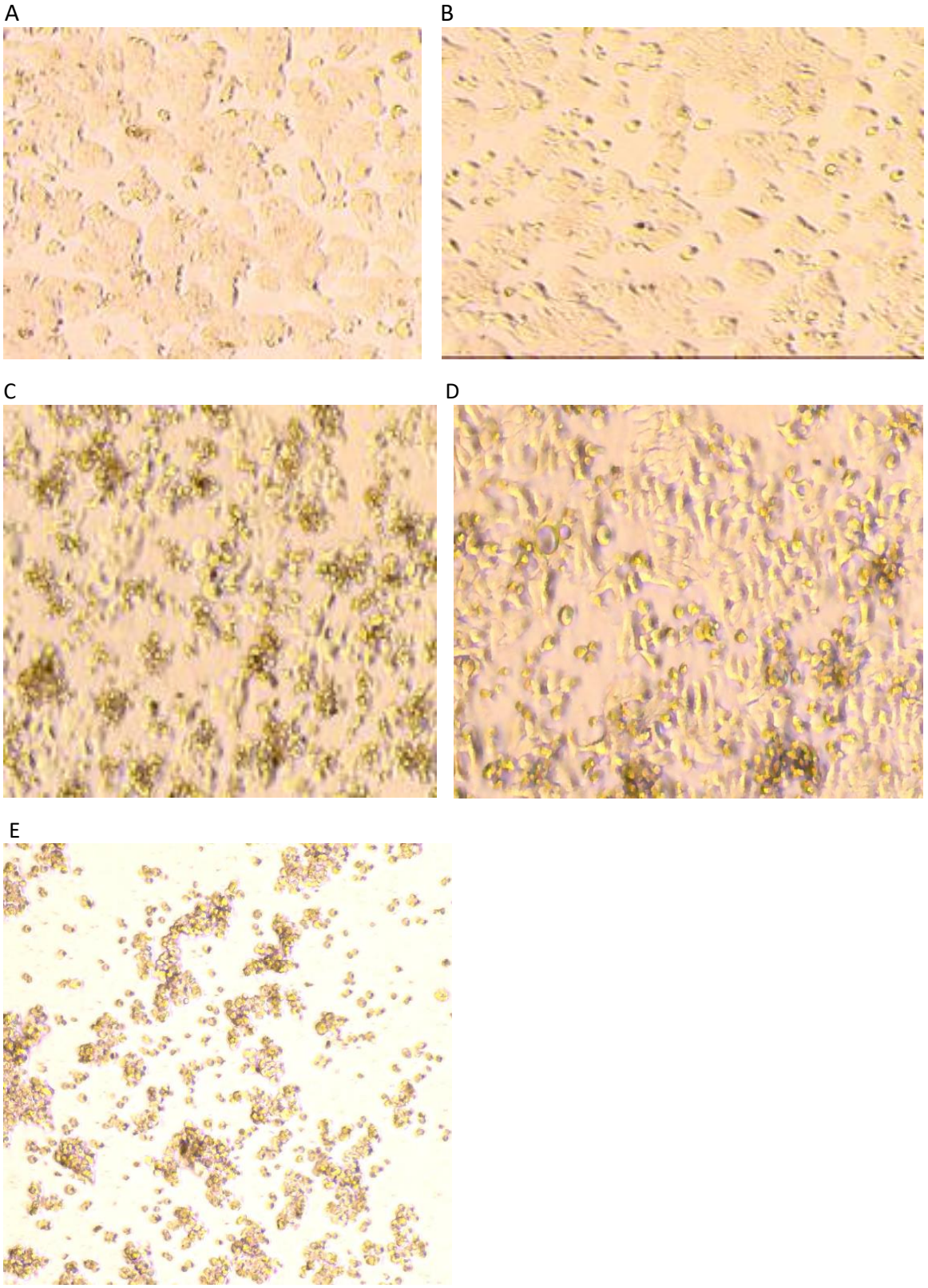


Fig: 6.3.1 Microscopic observation of HT-29 cell lines treated with p21 plasmid cDNA transfection at magnification of 20X objective (2048 × 1536 px). (A) Mock transfected control(B) vector-Lipofectamine control (C) p21-lipofectamine transfected(D)piffithrinμ and (E) pifithrin - μ combined p21 gene transfection.

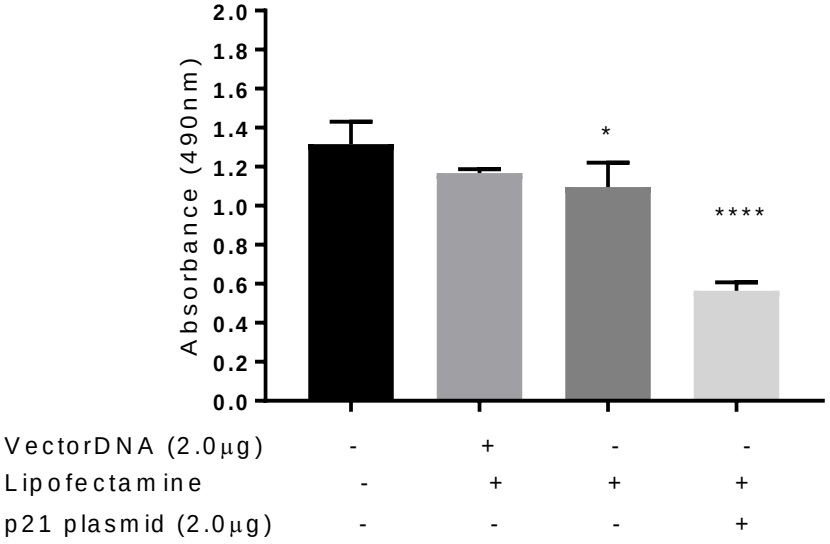


Fig: 6.3.2 Effect of p21-plasmid DNA transfection on HT-29 cell lines. HT-29 cell lines seeded onto 96 well assay plates at cell density (500,000 cells/ml) could adhere to the surface of the plates for 24 h. Cells were then treated with human p21 plasmid DNA incorporated in lipofectamine complex. The cells were incubated for 24 h in incubator at 37°C. Cell viability was measured by MTS assay. Data were analyzed statistically using one - way ANOVA, with post hoc Tukey's multiple comparisons test. Significant differences between control and transfected cell lines are indicated by * ($p<0.05$) mean±SD; n=4.

Table 6.1 Statistic presentation of Tukey's multiple comparisons test (ns* insignificant)

MTS Assay		
p21 plasmid DNA(2.0µg)	**Significance**	**P value**
Control vs lipofectamine control	*	P 0.0311
Control vs vector control	ns	P 0.1338
Control vs p21 plasmid DNA	****	P<0.0001
Lipofectamine control vs p21 plasmid DNA	****	P<0.0001
Vector control vs p21 plasmid DNA	****	P<0.0001

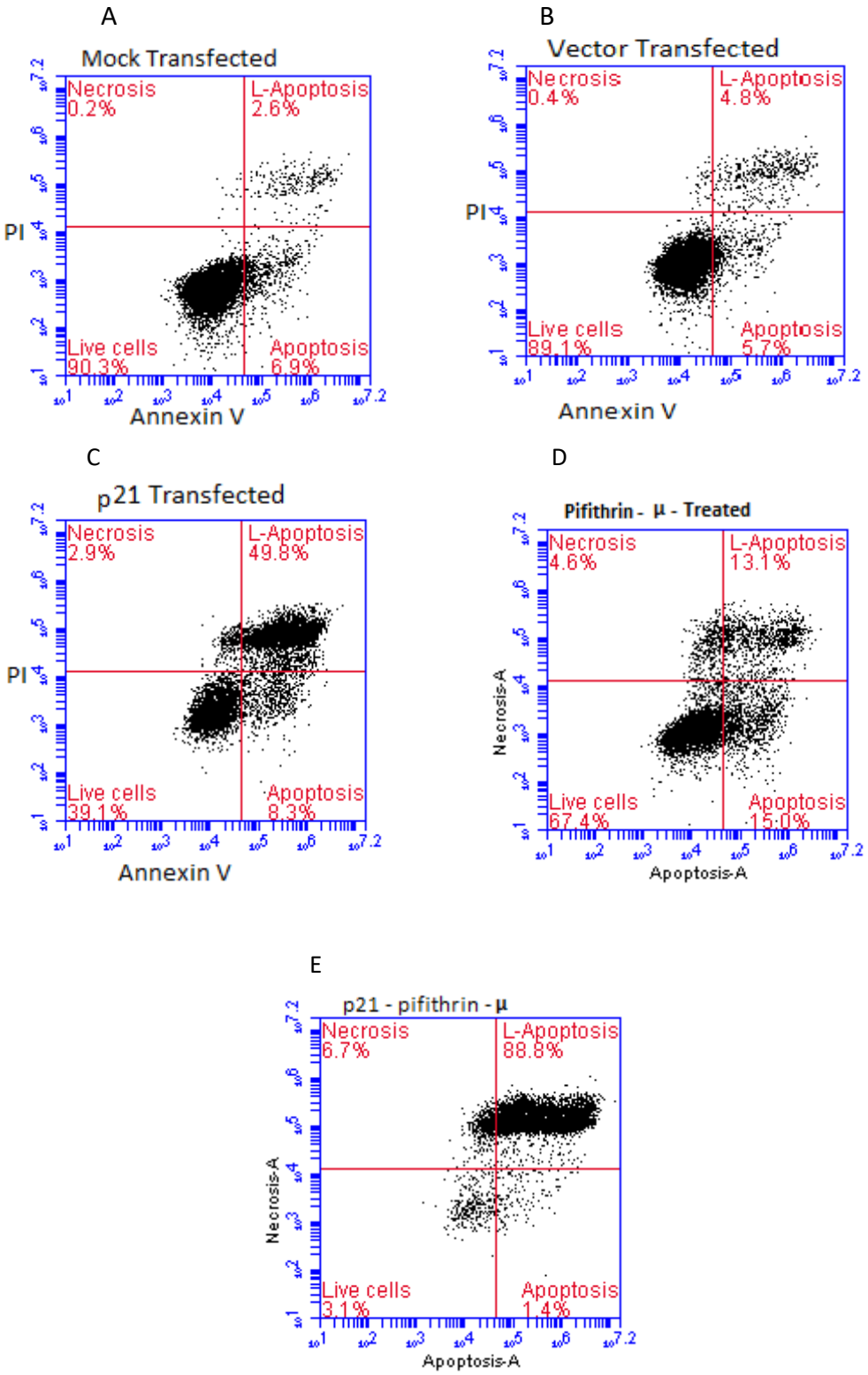


Fig: 6.3.3 Flowcytometric data presentation of annexin V and PI staining in HT-29 cell lines. HT-29 cell lines at cell density (1×10^6 cells), seeded onto 6 well assay plates were exposed to pifithrin - μ (15μM) followed by transfection with p21 plasmid DNA. The cells were allowed for incubation at 37◦C for 24h. The cells were then analyzed by annexin V and PI staining using flow cytometer- (A) unstained control, (B) Vector transfected, (C) p21 plasmid DNA transfected, (D) pifithrin - μ treated and (E) pifithrin -μ- p21 plasmid DNA.

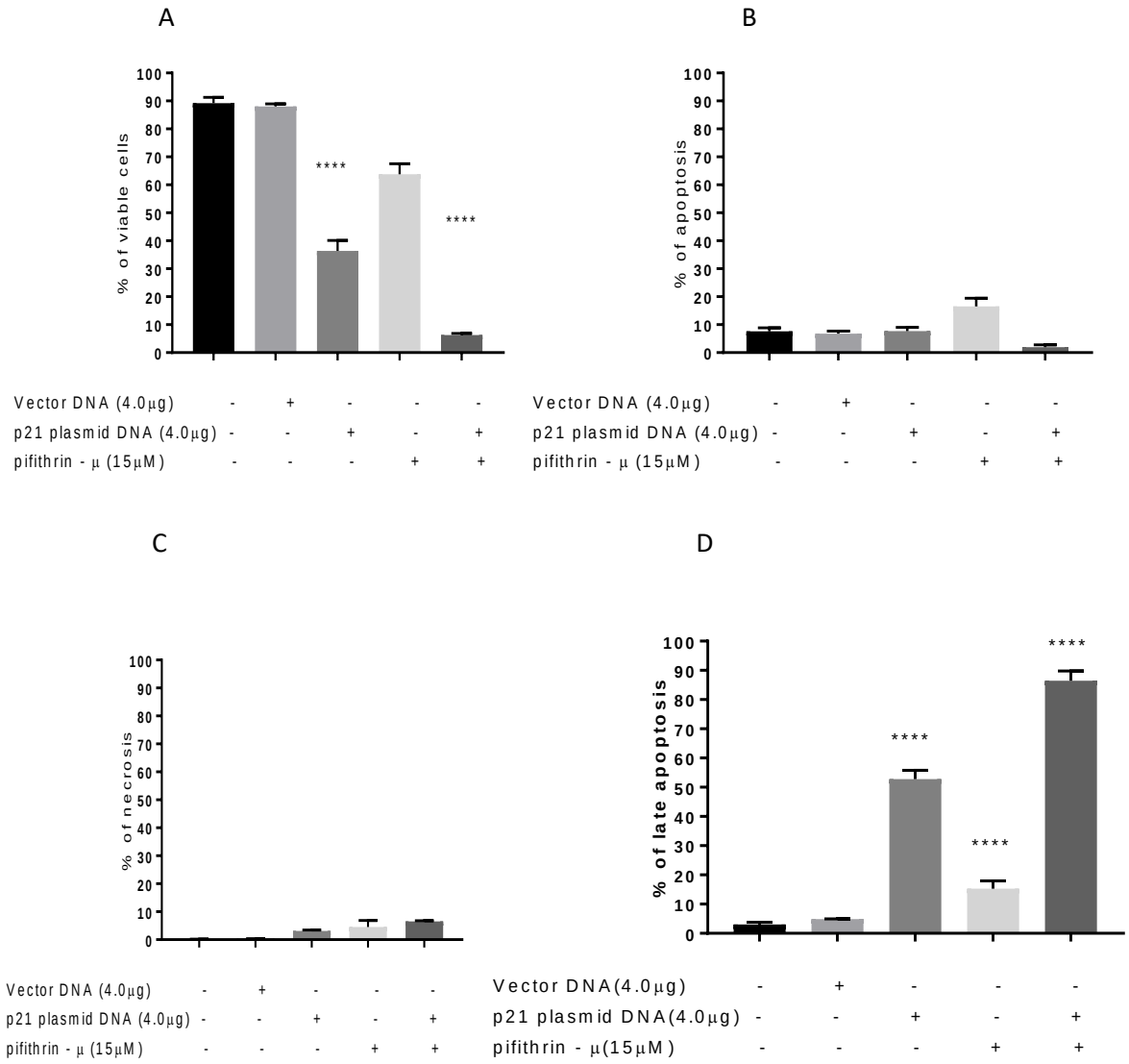


Fig: 6.3.4 Effect of p21 plasmid cDNA transfection determination by annexin V and PI staining. HT-29 cell lines seeded onto 6 well assay plates at cell density (1×10^6cells) could adhere to the surface of the plates for 24 h. Cells were then treated with human p21 plasmid cDNA - lipofectamine complex, vector DNA in lipofectamine independently and in combination with pifithrin-µ (15µM). The cells were incubated for 24 h in incubator at 37℃. Cell viability was measured by Annexin V and PI by flow cytometer. Data were analyzed statistically using one - way ANOVA, with post hoc Tukey's multiple comparisons test. Significant differences between control and transfected cell lines are indicated by * ($p<0.05$) mean±SD; n=4.

Table 6.2 Statistic presentation of Tukey's multiple comparisons test (ns* insignificant)

%of cell viability	Significance	P value
Control vs pifithrin - µ	****	P<0.0001
Control vs vector control	ns	P 0.9804
Control vs p21 plasmid cDNA	****	P<0.0001
Vector control vs p21 plasmid cDNA	****	P<0.0001
Pifithrin - µ vs p21 plasmid cDNA-pifithrin-µ		

% of apoptosis	Significance	P value
Control vs pifithrin - µ	*	P 0.0126
Control vs vector control	ns	P 0.9693
Control vs p21 plasmid cDNA	ns	P >0.9999
Vector control vs p21 plasmid cDNA	ns	P 0.9486
Pifithrin - µ vs p21 plasmid cDNA-pifithrin-µ	****	P <0.0001

% of necrosis	Significance	P value
Control vs pifithrin - µ	**	P 0.0069
Control vs vector control	ns	P 0.9994
Control vs p21 plasmid cDNA	ns	P 0.0572
Vector control vs p21 plasmid cDNA	ns	P 0.0787
Pifithrin - µ vs p21 plasmid cDNA-pifithrin-µ	ns	P 0.3590

% of late apoptosis	Significance	P value
Control vs pifithrin - µ	**	P 0.0069
Control vs vector control	ns	P 0.8388
Control vs p21 plasmid cDNA	****	P<0.0001
Vector control vs p21 plasmid cDNA	****	P<0.0001
Pifithrin - µ vs p21 plasmid cDNA-pifithrin-µ	****	P<0.0001

6.3.2 Evaluation of pifithrin - μ assisted inhibition of HSPA1A in colorectal cancer cell line HT-29

The HT-29 cell lines subjected to treatment with pifithrin -μ were analysed for HSPA1A protein inhibition using HSPA1A primary antibody and FITC labelled secondary antibody showed significant inhibition of HSPA1A with pifithrin - μ at 15μM concentration (Fig 6.3.5).

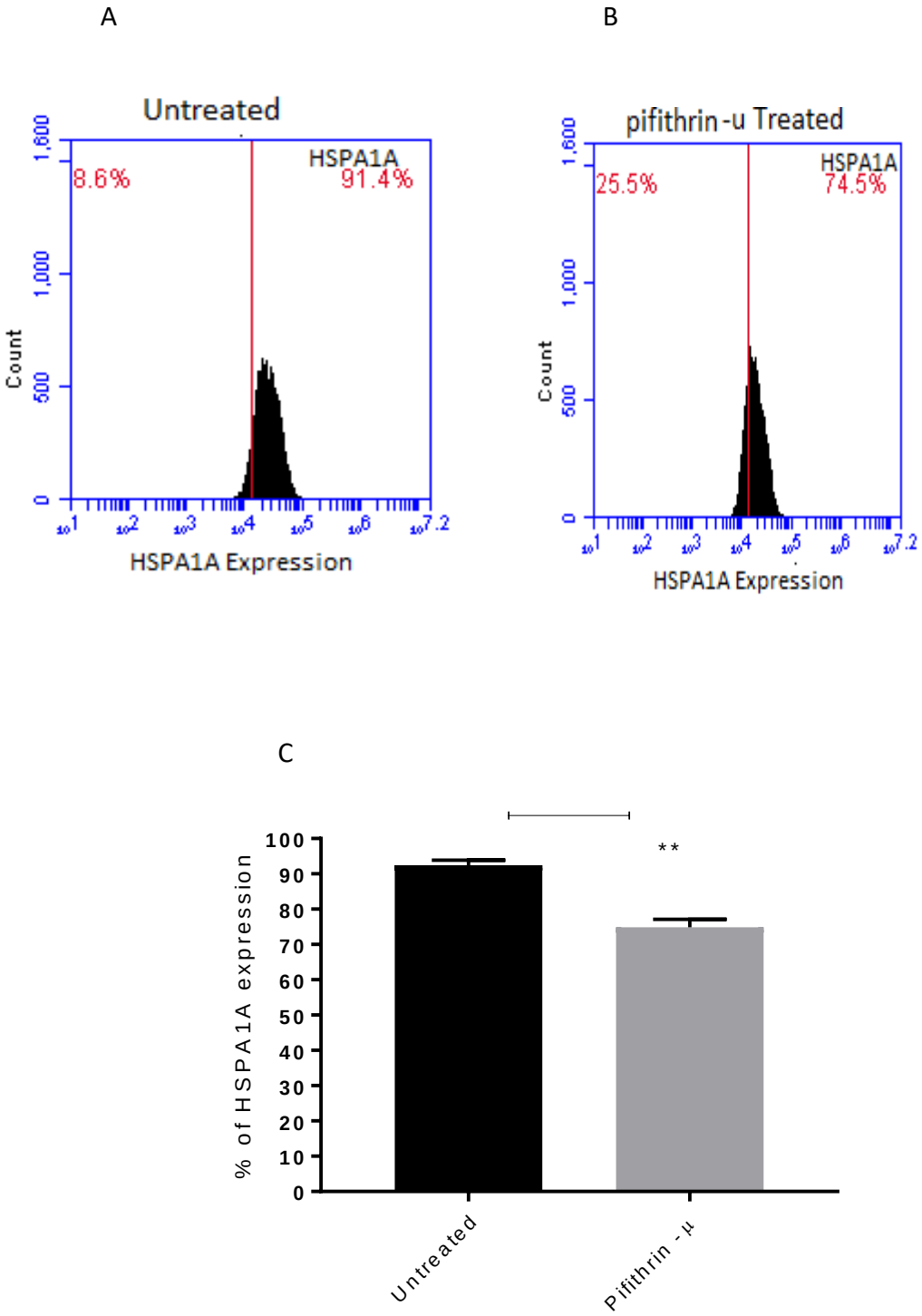


Fig: 6.3.5 Intracellular inhibition of pifithrin - μ facilitated HSPA1A protein expression in HT-29 colon cancer cell line HT-29 cell lines seeded onto 6 well assay plates at cell density (1×10^6cells) could adhere to the surface of the plates for 24 h. Cells were then treated with Pifithrin-μ(15μM). The cells were incubated for 24 h in incubator at 37°C. Cells were subjected to determination of HSPA1A protein expression by flow cytometer using FITC labelled secondary antibody. Data were analyzed statistically using paired t-test. Significant differences between control and treated cell lines are indicated by * ($p<0.05$) mean±SD; n=3, HSPA1A proteins(p0.099**).

6.3.3 Determination of intracellular and cell surface levels of p21 protein resulted from p21 plasmid DNA transfection by flow cytometer

The overexpression of p21 was analysed using FITC labelled p21 primary antibody showed no difference in expression in endogenous p21 levels with reference to untreated cell lines (Fig 6.3.6). However, p21 induction expressed a significant rise in expression of cell surface p21 protein 20.8% compared to the untransfected controls (Fig 6.3.7).

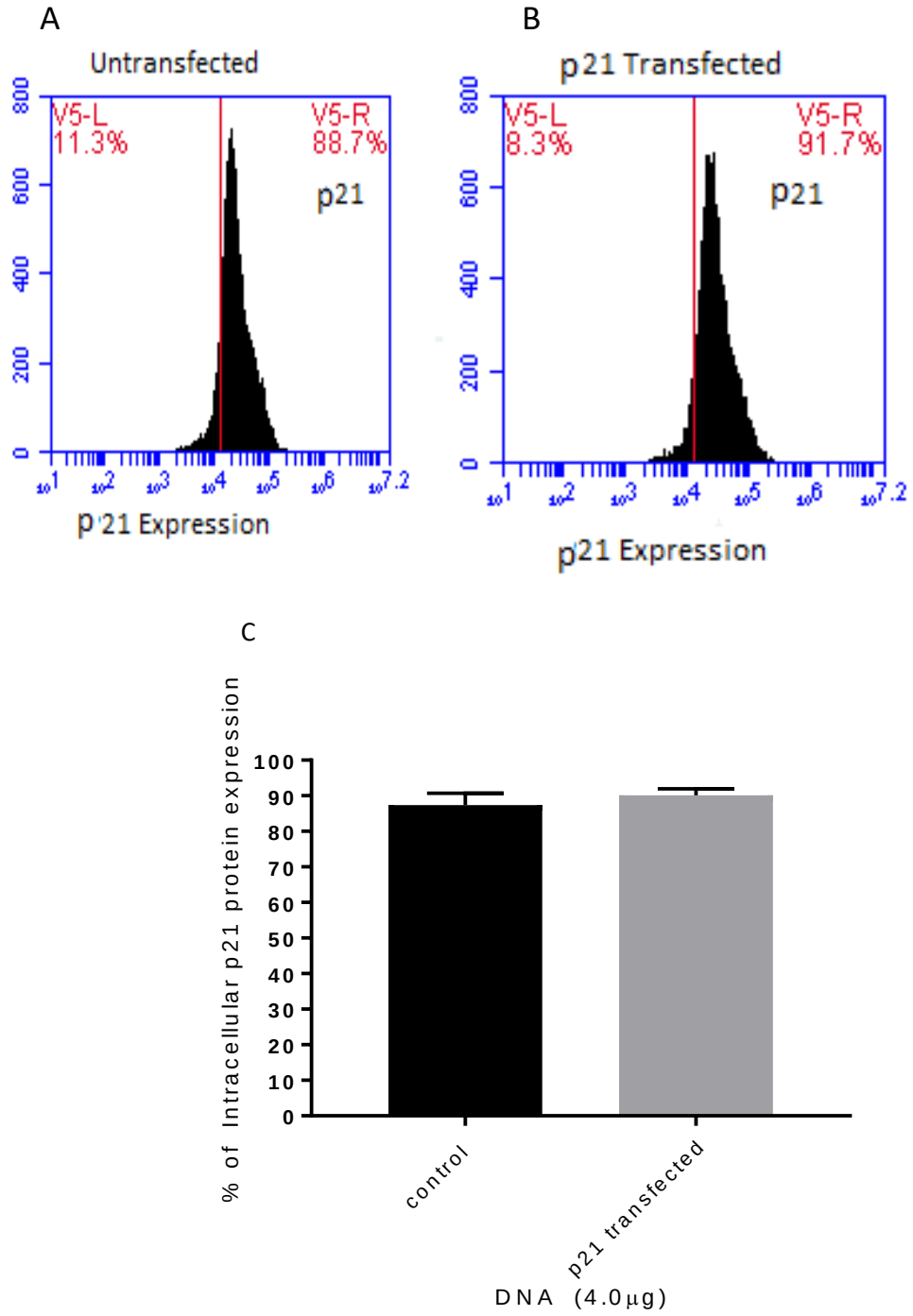


Fig: 6.3.6 Intracellular p21 protein expression in HT-29 colon cancer cell line HT-29 cell lines seeded onto 6 well assay plates at cell density (1×10^6 cells) could adhere to the surface of the plates for 24 h. Cells were then treated with p21 plasmid cDNA in lipofectamine. The cells were incubated for 24 h in incubator at 37°C. Cells were subjected to determination of endogenous p21 protein expression by flow cytometer using FITC labelled p21 primary antibody. Data were analyzed statistically using paired t-test showed insignificant differences between p21 levels in control and treated cell lines are indicated by * ($p<0.05$) mean±SD; n=3, p21 proteins(p0.1193).

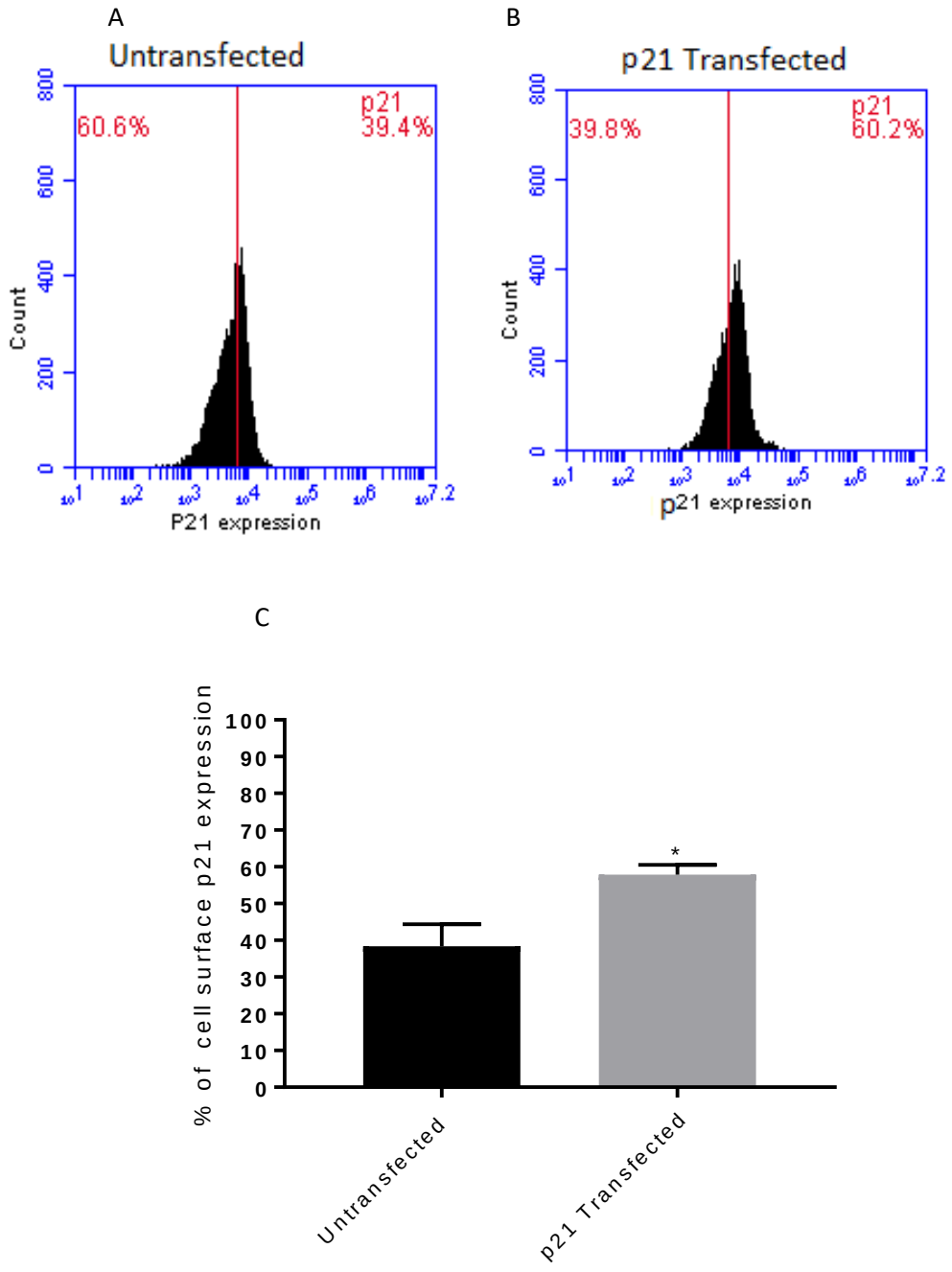


Fig: 6.3.7 p21 protein expression at cell surface in HT-29 colon cancer cell line. HT-29 cell lines seeded onto 6 well assay plates at cell density (1×10^6 cells) could adhere to the surface of the plates for 24 h. Cells were then treated with p21 plasmid cDNA in lipofectamine). The cells were incubated for 24h in incubator at 37^{o}C. Cells were subjected to determination of cell surface p21 protein expression by flow cytometer using FITC labelled p21 primary antibody. Data were analyzed statistically using paired t-test. Significant differences between control and treated cell lines are indicated by * ($p<0.05$) mean±SD; n=3, p21 proteins(p0.0163*).

6.4 Discussion

Over the years studies have shown the efficient role of p21 gene in accelerating programmed cell death due to elevated expression levels of this gene which in turn resulted in improved sensitivity towards conventional chemotherapeutics in a wide range of malignancies. Adenoviral mediated gene transfection studies support the efficiency of p21 gene in arresting the roots of colorectal progression and metastasis (Wang et al. 2015). p21 gene expression is studied to be accelerated in colorectal cancers by various pathways, one such route is MEK/ERK, which is counter regulated by transforming growth factor. TGF β works through SMAD signalling cascade boosting the level of p21 protein initiating cell cycle arrest (Bauer. et al. 2015; Padua. 2009).

Cancer cells are protected from undesirable survival conditions by genes of the heat shock family. These stress responsive chaperons possessing different molecular weights, are found elevated in cancer cells are evidenced to play an eminent role in angiogenesis and metastasis, demoting cellular death pathways involving caspase stimulation (Beere et al. 2000).The underlying molecular functions of heat shock proteins and their interactions with tumors suppressor proteins are still unclear and needed to be explored in depth.

This present study demonstrated that p21 overexpression induced apoptosis in the HT-29 colorectal cancer cell line and the levels of apoptosis were more pronounced when cells overexpressing p21 were treated with pifithrin - μ which inhibits HSPA1A (Fig 6.3.5). This study appears to be a novel finding. Hence HSPA1A proteins are showcased to be potential targets for successful p21 induced gene therapies for colorectal malignancies**.** Biopsy studies evidence increased expression of HSPA1A, which are observed to be intimately linked with tumors nodule assisted angiogenesis in relapsed colorectal cancers (Murphy 2013; Hwang et al. 2003). Hence inhibiting HSPA1A may enhance the sensitivity of cancer cells towards conventional therapies, improving survival rates.

Pifithrin - μ assisted apoptosis (Fig 6.3.3D) in HT-29 cell lines, studies show that Pifithrin - μ hampers the function of HSPA1A by directly binding to the C terminal domain of HSPA1A, in turn preventing its interaction with pro-apoptotic proteins of the mitochondrial apoptotic

signaling pathway and p53 gene resulting in promoting apoptosis in cancer cells (McKeon et al. 2016) . Studies reveals that, Pifithrin - μ administration hinders autophagy - which is a lysosome catalysed cellular mechanism, which plays a major role in discarding of unwanted metabolic residues within cancer cells thus, allowing successful proliferation. (Mizushima et al. 2008; Jones et al. 2016).

The colon cancer cell lines HT - 29 exhibited elevated levels of HSPA1A proteins with reference to pifithrin - μ treated cell lines which drastically inhibited HSPA1A intracellularly (Fig 6.3.5). Due to HSPA1A protein suppression, the cells expressed reduced cell viability, which predicts the eminent role of HSPA1A in colon cancer survival. As expected, the overexpression of p21 (Fig 6.3.3), reflected significant apoptosis in HT-29 cell lines, and the cell lines subjected to p21 over expression combined with pifithrin - μ assisted HSPA1A inhibition interpreted more pronounced effect with reference to respected controls. As the data controls for pifithrin - μ in lipofectamine has not been evaluated in this study. There are also chances of lipofectamine in the transfection medium to form complexes with pifithrin - μ, which may also account for enhanced apoptosis in P21 plasmid cDNA transfected HT-29 cell line. Moreover, as the lipofectamine transfection may have created pores in the cell membrane, there are chances for leakage of protein overexpressed to reach the cell surface, detecting enhanced p21 levels in the cell surface as compared to mock transfected controls as shown in (Fig 6.3.7), and this may account for the lack of overexpressed p21 at the intracellular levels.

Our results collectively interpret the active participation of tumor suppressor gene p21 in arresting the proliferation of colon cancer cell line HT-29 and also reflects the inhibitory status of HSPA1A proteins in enhancing p21 induced apoptosis in HT-29 cell lines (Archer et al. 1998; Yamamoto et al. 1999).

Chapter 7

7.1 Discussion and future research

In the current scenario, the increasing resistance together with the side effects produced are the major concerns encountered with conventional cancer treatments including chemotherapy and radiation. Hence the need to develop potentially targeted therapies, with fewer side effects is a priority. Hence, considering the interactive role of the stress protein HSPA1A in arresting cancers together with the supporting evidence from previous *in vitro* and *in vivo* studies stating the therapeutic potential of tumour suppressor gene p21 in arresting the growth of colorectal cancer cell lines. This thesis aimed at studying a two - pronged approach, to evaluate the effects of p21 protein overexpression in combination with HSPA1A inhibition in colorectal cancer cell lines HT-29.

It is also important to note that all experiments were performed with cancer cell lines maintained in culture medium devoid of antimycotic /antibiotics. The results from chapter 3 shows the involvement of these agents in inducing the expression of HSPA1A at extracellular levels in colorectal cancer cell lines. And these proteins may interfere with the protein translational processes in cancer cells, thus affecting their normal proliferation. Hence, reports highly denounce antimycotic /antibiotic application for *in vitro* investigations (Kuhlmann 1995; Llobet et al. 2015). The thesis looked at two different cancer cell lines in chapter 4 - leukaemia cancer cell lines U937 and colorectal cancer cell lines HT-29 to evaluate how the role of HSPA1A inhibitor pifithrin - μ or role of HSPA1A in hyperthermia differ with their inhibitory status in sustaining cancer growth in the two different cancer types. And the results from this chapter provided an understanding of the inhibitory effect of HSPA1A proteins, which accelerated UV radiation induced apoptosis.

The insensitiveness to widely accepted treatment approaches is a major issue in cancer treatments. In case of radiotherapy, prolonged exposures may lead to undesirable effects to the healthy tissues. Hence studies at the molecular level are trying to modulate radiotherapy to display its highest efficiency with limited range of exposures. Previous report had revealed the inhibitory activity of HSPA1A proteins using pifithrin isoforms α and β in gama radiation induced cancer arrest (Walton et al. 2005). However, the apoptotic role of pifithrin - μ

mediated inhibition of HSPA1A proteins in UV induced apoptosis addressed in chapter 4 has not been explored previously. Hence the first phase of this thesis initially addressed the interaction of pifithrin - μ mediated HSPA1A protein inhibition in combination with UV radiation, which showcased accelerated rate in apoptosis induced by UV radiation at limited exposure durations in leukaemia cell lines U937 and colorectal cancer cell lines HT-29. Thus, suggesting that, resistance to therapies could be tackled by implementing drugs targeting HSPA1A proteins as a combination treatment.

Though, as previously stated the guarding role of HSPA1A proteins, which function in binding to and re-establishing the functional status of impaired proteins, thus prevented cell cycle arrest, resulting in cancer cell survival. Moreover, studies have also reflected the crucial role of HSPA1A in inducing resistance to conventional therapies (Sherman. 2015; Daugaard et al. 2005), however, their escalated expression is studied to communicate with the host natural defence mechanism in treatments such as hyperthermia in cancers in solid malignancies (Urano et al. 1983). Previous studies have displayed hyperthermia effects in colorectal cancers *in vivo*, but the role of HSPA1A proteins due to hyperthermia treatment in colorectal cancer cell line HT-29 has not been revealed. Hence this study evaluated the role of HSPA1A in hyperthermia study in both the cancer cell lines, thus showcasing apoptosis with hyperthermia at 42℃. This fluctuation in temperature also triggered the expression of HSPA1A proteins to escalate. Studies relate the increased expression of HSPA1A proteins in acting as signals for activating natural host defence mechanism against cancer survival (Jolesch et al. 2012).

The second phase of the thesis chapter 5 focussed in selecting a non - cytotoxic transport system for the safe delivery of p21 plasmid cDNA in colorectal cancer cell lines HT-29 for p21 overexpression and apoptosis. *In vitro* transfection studies rely on electroporation method, nanoparticles or microparticles for intracellular delivery of biological entities such as plasmid DNA or siRNA, as the cellular mucosal membranes act as barriers, thus hampering the exchange of macro molecules. As the particles of micro size and smaller are studied to be engulfed by the process of endocytosis, they are widely used for *in vitro* and *in vivo* experiments. Although studies highlight the therapeutic potential of chitosan-based delivery systems (Hejazi. 2003; Bernkop et al .2012; Katas et al. 2013) in terms of its antioxidant properties, biodegradable biocompatible nature, thus making it extraordinary for drug

delivery applications, several other *in vitro* and *in vivo* reports evidence the side effects and toxic effects of chitosan (Qi. 2006; S. Wimardhani et al. 2014; Zhao et al. 2017).

Since, previous research evidence differing opinions in terms of cytotoxicity of chitosan delivery systems, with studies highlighting the cytotoxic effect of the system in its drug free state in a dose dependent manner on one side (Depani et al. 2013), and others suggesting it's non - cytotoxic nature (Qi. 2006; S. Wimardhani et al. 2014). This present study investigated chitosan microparticles and microgels crosslinked with glutaraldehyde or β - glycerol phosphate on colorectal cancer cell lines HT-29, using 5 - fluorouracil for cytotoxicity studies in HT-29 cancer cell lines.

Although these microgels or microparticles expressed enhanced cytotoxicity with 5 - fluorouracil compared to its independent cytotoxicity, these formulations were shown to exert cytotoxicity resulted from cross linking agents glycerol phosphate or glutaraldehyde used to graft the systems. Hence, the study concluded chitosan - based systems unsuitable for use as delivery system due to the cytotoxicity exerted by these systems. Besides chitosan, BSA formulated microparticles thus examined for cytotoxicity evaluation were also found to be unfit due to the cytotoxicity exerted by these particles independently. However, the thesis did not investigate the cytotoxic effect of these delivery systems in non - cancerous tissues, hence it is difficult at this stage to reveal a complete evaluation of the particles safety in medical applications. Hence in this study, the p21 gene inserted plasmid cDNA was delivered for transfection studies in colorectal cancer cell lines HT-29 using lipofectamine 2000 which is an efficient transfection system highly recommended for the delivery of biological entities for *in vitro* experimental studies (Li et al. 2015).

As described earlier, the prominent role of p21 in interacting with several apoptosis accelerating genes in arresting the cells from further replication, the functional status of p21 gene as a tumour suppressor is studied to vary with different cancer treatment strategies and some cancer types. Although reports evidence it's negative role in promoting cancer survival in treatments such as cryoinjury in colorectal cancer cell lines HT-29 and identified as the main cause of insensitiveness towards adjuvant therapeutic approaches involving chemotherapeutics and radiation therapies in patients battling with rectal cancers (Rau et al. 2003). However, the tumour suppressive status of p21 genes is a core factor in reflecting

sensitiveness to chemotherapeutics (Ravizza et al. 2004), and in playing an influential role in directing cancer cells to induce apoptosis.

Although previous studies have explored the therapeutic application of ectopic expression of the tumour suppressor gene p21, which reflects the active participation of p21 gene in initiating cell cycle arrest, thus demoting cancer survival in several malignancies (Yamamoto et al. 1999). Studies have not yet revealed the interactive role of HSPA1A ablation and tumour suppressor gene p21 overexpression in colorectal cancer cell lines or other cancers. Previous studies have demonstrated the active participation of p21 gene in inducing apoptosis (Archer et al. 1998; Yamamoto et al. 1999) which is in an agreement with the findings presented in this thesis in colorectal cancer cell lines HT-29.

Besides the role of tumour suppressive gene p21, the thesis also discussed pifithrin -μ assisted inhibitory roles of HSPA1A proteins in response to, radiation therapy and hyperthermia. The HSP 70 gene family are classified into the following genes, HSPA1A-, HSPA2, HSPA5, HSPA6, HSPA7, HSPA8, HSPA9, HSPA13, HSPA12A, HSP12B, HSPA14, which have a variety of individual functional roles in human cells. Intriguingly, studies proved escalated levels of HSPA1A genes related to cancer progression in most of the cancer malignancies. The HSP 70 genes plays a crucial role in re - establishing the functional status of proteins damaged due to oxidative stress response in cancer cells. They are also studied to hamper the mitochondrial apoptotic signalling by introducing a conformational alteration of the procaspase - 9, thus preventing the interaction of procaspase - 9 with apoptotic activating factor 1, eventually protecting cells from apoptosis (Daugaard et al. 2005). Studies also display the defensive mechanism of stress protein HSPA1A in protecting cancer cells from any extrinsic or intrinsic physiological stress (Klink et al. 2012; Goliaei. 1998). This defensive role exerted by HSP 70 family genes are reported to be regulated by blocking its intracellular interaction with NEF - nucleotide exchange factors and TPR - tetratricopeptide chains, which works in close connection with HSPA1A protein resulting in insensitiveness to natural host defense, promoting cancer survival (Assimon et al. 2013). Hence a deep understanding of the molecular mechanisms by which these proteins function in cancer cells, may help to improve the therapeutic index of conventional therapies (Kabakov et al. 2006).

Although HSPA1A proteins function in defending cancer cells from undergoing apoptosis, their functional status is studied to be important in treatments like hyperthermia, as the

increased presence of these proteins aid in triggering the natural defense mechanism which in turn arrest cancer cells according to previous reports (Milani V. 2002). Therefore, attempts of ectopic overexpression of HSPA1A genes have previously evidenced pronounced cancer arrest with cancer treatments like hyperthermia, thus outlining the positive response of these molecules in cancer attenuation as well. Several studies also reveal the inhibition of HSPA1A proteins as useful strategy which enhanced sensitivity of cancer cells towards several chemotherapeutics (Demidenko et al. 2005; Chakraborty et al. 2008; Fang et al. 2013).

Although HT-29 cell lines express p21 proteins as shown in chapter 6, these proteins found may be malfunctional to induce apoptosis. Moreover, there are chances for cancer cell expressive stress protein HSPA1A to bind with the available p21 and arrest its function, which may attribute to the insensitivity produced towards several chemotherapeutics and this may account for apoptotic activity observed upon treatment with pifithrin - μ mediated inhibition of HSPA1A proteins in P21 plasmid cDNA transfected HT-29 cell lines in this thesis.

Interactive role of HSPA1A protein in p21 plasmid cDNA transfection

The inhibitory status of HSPA1A in p21 plasmid cDNA transection study suggests the interaction of HSPA1A protein and tumour suppressor protein p21. The p21 protein expressed was refrained from undergoing apoptosis due to the high levels of HSPA1A proteins produced in cancer cell lines as displayed in (Fig 7.1.). The HSPA1A proteins bind with apoptotic proteins, hampering their transcription. Although, previous studies have revealed the direct interaction of tumour suppressor gene p53 with HSPA1A in accelerating p53 directed apoptosis in cancer cells (Elengoe et al. 2015). No studies have yet elucidated the interaction between tumour suppressor protein p21 and HSPA1A proteins.

Moreover, reports have highlighted the active role of HSPA1A proteins with several cellular components to fulfil its function as cancer promoter. They are studied to attach with lipid molecules, which upon binding, assist in delivering HSPA1A within intracellular destinations (Mccallister et al. 2015). Studies have also, evidenced the prominent role of HSPA1A protein upon attachment with the anti - apoptotic gene BAG 3 in promoting cancer growth by demoting the expression of several apoptotic inducing machinery including tumour suppressor gene p21 in *in vitro* studies in malignancies of breast and brain (Colvin et al. 2014; Li et al. 2015). Furthermore, induced transcription of p21 gene was illustrated in a two -

pronged *in vitro* treatment approach in which HSPA1A proteins were ablated prior to hyperthermia treatment (Sekihara et al. 2013). In addition to suggesting the interactive role of HSPA1A proteins with the tumour suppressor gene p21, this previous study also reported the inhibitory activity of HSPA1A proteins mediated by pifithrin - μ which accelerated the efficacy of hyperthermia treatment (Sekihara et al. 2013). The high expression of HSPA1A proteins were also associated with downregulation of p21, which enhanced the survival of breast and brain tumors (Rohde et al. 2005).

As a means of extrinsic stress response due to plasmid cDNA transfection, the expression of HSPA1 proteins tend to increase dramatically. The p21 plasmid cDNA reaching the destination may be affected by HSPA1A proteins, hampering a complete apoptotic activity as illustrated in (Fig 7.2). p21 plasmid cDNA induced apoptosis to around only 60% of HT-29 cell lines**.** Upon incorporating HSPA1A inhibitor pifithrin -μ, the inhibitor binds to HSPA1A and thereby prevents its interaction with the tumour surressor proteins p21 and p53, thus allowing their normal cell cycle regulatory functions, resulting in growth arrest of colorectal cancer cell lines HT-29. Apart from tumour suppressor proteins, transcription factors including BAX and death receptors will be free from HSPA1A activity, thus promoting the apoptotic pathway.

Hence, considering these supporting evidences from previous studies, finally suggesting the anti - apoptotic interaction of HSPA1A proteins that may account for the enhanced apoptosis resulted from HSPA1A inhibition in p21 plasmid cDNA transfection in HT-29 cancer cell lines, as presented in this thesis. To recapitulate, the inhibition of HSPA1A prior to p21 gene therapies may useful for successful gene therapy. As hypothesized, the p21 overexpression induced apoptosis on HT-29 cell lines and pifithrin - μ assisted HSPA1A protein inhibition set the p21 protein free from HSPA1A protein binding, thus allowing a healthy apoptotic pathway in colorectal cancer cell lines HT-29 (Fig 7.2), thus revealing a more promising therapeutic approach in p21 induced gene therapies for colorectal cancers.

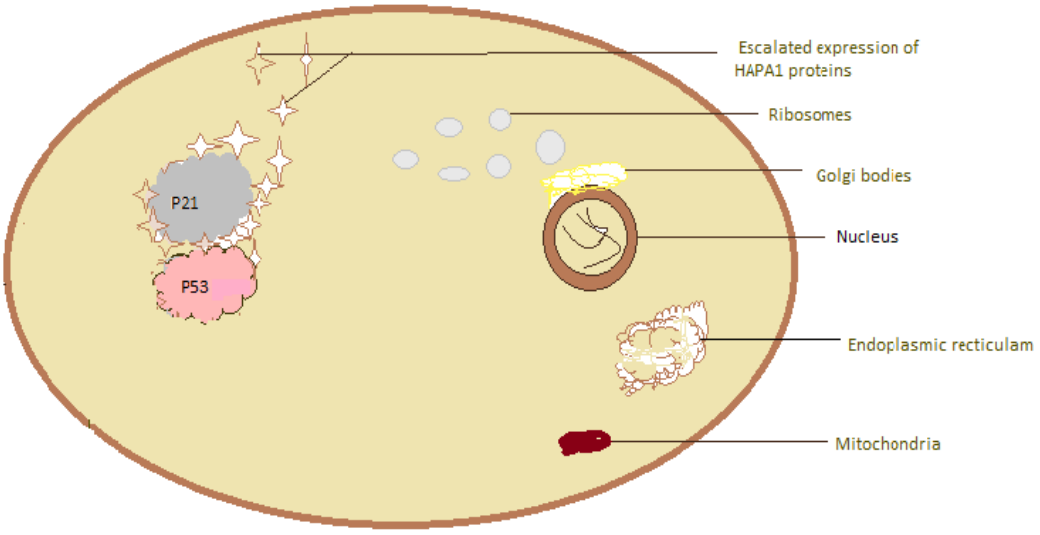


Fig 7.1 Interference of HSP A1A in cancer cells. The diagram shows the escalated expression of HSPA1A normally in cancer cell lines which interacts with apoptotic signaling factors p53 and p21, hampering their functional roles thus promoting cancer survival. Picture adapted from the data presented in this thesis and (Rohde et al. 2005; Demidenko et al. 2005; Chakraborty et al. 2008; Fang et al. 2013).

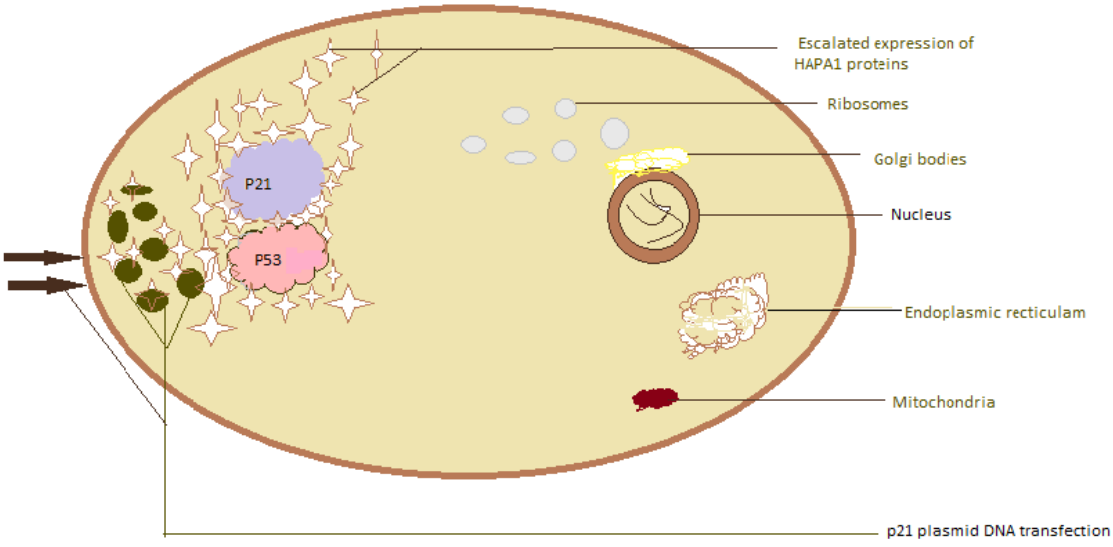


Fig 7.2 Interference of HSPA1A in p21 transfection. The diagram shows that cancer cells may experience cellular stress due to p21 plasmid DNA transfection, which in turn result in overexpression of HSPA1A. The overexpressed HSPA1A could bind to p21 plasmid DNA and may hamper the transfection and p21 expression and prevent apoptosis. Picture adapted from the data presented in this thesis and (Rohde et al. 2005; Demidenko et al. 2005; Chakraborty et al. 2008; Fang et al. 2013).

Future studies of this present study highlight the following routes yet to be investigated

- Although this thesis suggests the interactive binding of the HSPA1A proteins to p21 proteins as one of the reasons which maybe hampering the complete apoptotic effect through p21 plasmid DNA transfection. However, this can be confirmed only through protein - protein interaction studies. Hence, this needs to be further confirmed using far western blotting technique or fluorescent microscopic observations. As an initial phase of this future study, untreated colorectal cancer cell line HT-29 should be explored for the HSPA1A - p21 molecular binding, to understand the binding profiles of HSPA1A to tumour suppressors p21 or p53. Secondly, the protein - protein interactions studies needs to be explored with the incorporation of HSPA1A inhibitor pifithrin - μ, to ascertain its activity in arresting cancer progression. Finally, the interaction of HSPA1A with the ectopically expressed p21 proteins needs be addressed. As this may provide an explicit insight of how p21 induced gene therapies could be boosted to achieve its maximum treatment efficiency through inhibition of HSPA1A proteins. Moreover, the interactive role tumour suppressor protein p53 also needed to be evaluated in p21 induced gene therapies as p21 mediated apoptosis also relies on the functional mode of p53 gene.

- Adjuvant gene therapies using p53 and p21, through inhibition of HSPA1A proteins needs to be further investigated as it could be useful therapeutic strategy in cancers with mutant p53 or p21 genes.

- Hyperthermia is an effective therapy (Jolesch et al. 2012; Urano et al. 1983). The role of hyperthermia along with tumour suppressor gene therapies is yet to revealed. Although HSPA1A proteins are considerably triggered by hyperthermia, as this protein play a major part in signalling the immune system, this defensive role of these unique proteins may in turn boost the therapeutic efficiency of p21 gene induced gene therapy in cancers with mutated p21 genes.

- The antibiotics works as efficient anti - cancer agents (De Francesco et al. 2017). The side effects of these agents as discussed previously can be minimised by engineering these compounds into nanoparticle size as this may provide targeted delivery, thus improving the therapeutic efficacy even with low dosage administration. This thesis

shows the escalated expression of HSPA1A protein resulted from anti - mycotic antibiotic treatment and with hyperthermia treatment. Hence besides independent effects, a combined therapeutic effect of both these treatments may be good therapeutic model for several cancers, those including mutations in apoptotic operons.

- Although the thesis displayed enhanced apoptotic effects with UV radiation with the aid of HSPA1A inhibitor compound pifithrin - μ. The effects of UV radiation could be improved by inducing the expression of HSPA1A proteins by administration of treatments such as hyperthermia, which may result in triggering the host immune activity and maybe a useful cancer treatment approach to be investigated in future.

CHAPTER 8

References

Abbas, T. and A. Dutta (2009) "p21 in cancer: intricate networks and multiple activities"; Nat Rev Cancer 9(6): 400-414.

Ahmed K, Zaidi S F,(2013) “Treating cancer with heat; Hyperthermia as promising strategy to enhace apoptosis” ; J,Park Med Assoc Pubmed 23905451.

Addo, R. T., K. G. Yeboah, R. C. Siwale, A. Siddig, A. Jones, R. V. Ubale, J. Akande, H. Nettey, N. J. Patel, E. Addo and M. J. D'Souza (2015). “Formulation and Characterization of Atropine Sulfate in Albumin–Chitosan Microparticles for In Vivo Ocular Drug Delivery." Journal of Pharmaceutical Sciences 104(5): 1677-1690.

Archer, S. Y., S. Meng, A. Shei and R. A. Hodin (1998).” p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells”. Natl Acad Sci U S A 95(12): 6791-6796.

Amer.H;(2014).”Gene therapy for cancer-present status and future perspective” pubmed:4452068.

Anon- (2002) “Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease”. Lancet, 360, 187-95.

Anders Abarrategi, Ana Civantos, Viviano Ramos, Jose Vicente Sanz Casado and Jose Luis (2008); “Chitosan Film as rhBMP2 Carrier: Delivery properties for bone tissue application”; Bio macro molecules 9,2,711-718.

Antonio, E., N. M. Khalil and R. M. Mainardes (2016).” Bovine Serum Albumin Nanoparticles Containing Quercetin: Characterization and Antioxidant Activity”. J Nanosci Nanotechnol 16(2): 1346-1353.

Ahsan, S. M., Thomas, M., I. (2017). “Chitosan as Biomaterial in Drug Delivery and Tissue Engineering”. International Journal of Biological Macromolecules:280.

Assimon, V. A., A. T. Gillies, J. N. Rauch and J. E. Gestwicki (2013). "Hsp70 protein complexes as drug targets." Curr Pharm Des 19(3): 404-417.

Artursson P;(1994); “Effect of chitosan on the permeability of monolayers of Intestinal Epithelial cells (Caco-2)”; Pubmed: 7816770.

Axelrod, D. E., S. Vedula and J. Obaniyi (2017). "Effective chemotherapy of heterogeneous and drug-resistant early colon cancers by intermittent dose schedules: a computer simulation study." Cancer Chemother Pharmacol 79(5): 889-898.

Azuma, K., T. Osaki, S. Minami and Y. Okamoto (2015). "Anticancer and Anti-Inflammatory Properties of Chitin and Chitosan Oligosaccharides." Journal of Functional Biomaterials 6(1): 33.

Bauer, J., O. Ozden, N. Akagi, T. Carroll, D. R. Principe, J. J. Staudacher, M. E. Spehlmann, L. Eckmann, P. J. Grippo and B. Jung (2015).” Activin and TGFβ use diverging mitogenic signaling in advanced colon cancer”. Molecular Cancer 14: 182.

Bae, I. H., Wook Kang, S., Hwan Yoon, S. & Um, H.-D. (2006).” Cellular components involved in the cell death induced by cisplatin in the absence of p53 activation”. Oncology reports:1175-1180.

Beere, H. M., B. B. Wolf, K. Cain, D. D. Mosser, A. Mahboubi, T. Kuwana, P. Tailor, R. I. Morimoto, G. M. Cohen and D. R. Green (2000). “Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome”. Nat Cell Biol 2(8): 469-475.

Benisty H, Violeta Beltran, Julia Burnier, Imre Berger, Luis Serrano, and Christina K (2015). “Tuneable endogenous mammalian target complementation via multiplexed plasmid based recombineering”; pubmed: 4661934.

Bernkop-Schnurch, A. and S. Dunnhaupt (2012).” Chitosan-based drug delivery systems”. Eur J Pharm Biopharm 81(3): 463-469.

Boige, Mendiboure et al. (2010)” Pharmacogenetic Assessment of Toxicity and Outcome in Patients with Metastatic Colorectal Cancer Treated with LV5FU2, FOLFOX, and FOLFIRI: FFCD 2000-05”. Journal of Clinical Oncology 28(15): 2556-2564.

Bock C H, Schwartz A G, Ruterbusch, Levin A M, Neslund – Dudas C, Wenzalaff A S, Reich D, McKeique P, Chen W, Heath EI, Powell Ii, Kittles R A, Rybicki B A (2009) “ Results from a prostate cancer admixture mapping study in African- American men”; pubmed: 19568772.

Boursi, B., Mamtani, R., Haynes, K. & Yang, Y.-X. (2015) “Recurrent antibiotic exposure may promote cancer formation - Another step in understanding the role of the human microbiota?” European Journal of Cancer, 51, 2655-2664.

Bañobre-López, M., Teijeiro, A. & Rivas, J. (2013).” Magnetic Nanoparticle-Based Hyperthermia for Cancer Treatment”. Reports of Practical Oncology & Radiotherapy, 18, 397-400.

Bhattarai, N., Gunn, J. & Zhang, M. (2010). “Chitosan-Based Hydrogels for Controlled, Localized Drug Delivery”. Advanced Drug Delivery Reviews, 62, 83-99.

Bloomston, M., Zervos, E. E. & Rosemurgy, A. S. (2002). “Matrix Metalloproteinases and Their Role in Pancreatic Cancer: A Review of Preclinical Studies and Clinical Trials”. Annals of Surgical Oncology, 9, 668-674.

Buzea C, Pacheoll, Robbie K(2007); “Nanomaterials and nanoparticles: sources and toxicity”; (Pubmed). PMID:20419892.

Cao, Y., Wu, K., Mehta, R., Drew, D. A., Song, M., Lochhead, P., Nguyen, L. H., Izard, J., Fuchs, C. S., Garrett, W. S., Huttenhower, C., Ogino, S., Giovannucci, E. L. & Chan, A. T.(2017).” Long-term use of antibiotics and risk of colorectal adenoma”. Pubmed :454611.

Campbell, M. J., G. S. Reddy and H. P. Koeffler (1997). “Vitamin D3 analogs and their 24-oxo metabolites equally inhibit clonal proliferation of a variety of cancer cells but have differing molecular effects”. J Cell Biochem 66(3): 413-425.

Cappuzzo, F., F. R. Hirsch, E. Rossi, S. Bartolini, G. L. Ceresoli, L. Bemis, J. Haney, S. Witta, K. Danenberg, I. Domenichini, V. Ludovini, E. Magrini, V. Gregorc, C. Doglioni, A. Sidoni, M. Tonato, W. A. Franklin, L. Crino, J. P. A. Bunn and M. Varella-Garcia (2005). “Epidermal Growth Factor Receptor Gene and Protein and Gefitinib Sensitivity in Non–Small-Cell Lung Cancer”. JNCI: Journal of the National Cancer Institute 97(9): 643-655.

Casa, D. M., T. K. Karam, C. Alves Ade, A. A. Zgoda, N. M. Khalil and R. M. Mainardes (2015). “Bovine Serum Albumin Nanoparticles Containing Amphotericin B: Characterization, Cytotoxicity and In Vitro Antifungal Evaluation”. J Nanosci Nanotechnol 15(12): 10183-10188.

Chatterjee, S. & Burns, t. f. (2017). “ Targeting heat shock proteins in cancer: a promising therapeutic approach”. international journal of molecular sciences, 18, 1978.

Ciocca & Calderwood, S. K. (2005).” Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications”. cell stress & chaperones, 10, 86-103.

Ciofani, G., Raffa, V., Menciassi, & Dario, p. (2008).” Alginate and chitosan particles as drug delivery system for cell therapy”. biomedical microdevices, 10, 131-140.

Chen, Y., Z. Yakhini, A. Ben-Dor, E. Dougherty, J. M. Trent and M. Bittner (2001). “Analysis of expression patterns: the scope of the problem, the problem of scope”. Dis Markers 17(2): 59-65.

Cheng, Y.-H., Riviere, J. E., Monteiro-Riviere, N. A. & Lin, Z. (2018). “Probabilistic risk assessment of nanoparticles after intravenous administration by integrating in vitro and in vivo toxicity with physiologically based pharmacokinetic modeling”. Nanotoxicology, 12, 453-469.

Chua, B. Y., M. Al Kobaisi, W. Zeng, D. Mainwaring and D. C. Jackson (2012). Chitosan Microparticles and Nanoparticles as Biocompatible Delivery Vehicles for Peptide and Protein-Based Immunocontraceptive Vaccines. Molecular Pharmaceutics 9(1): 81-90.

Chakraborty, P. K., S. B. Mustafi, S. Ganguly, M. Chatterjee and S. Raha (2008). “Resveratrol induces apoptosis in K562 (chronic myelogenous leukemia) cells by targeting a key survival protein, heat shock protein 70”. Cancer Science 99(6): 1109-1116.

Colvin, T. A., V. L. Gabai, J. Gong, S. K. Calderwood, H. Li, S. Gummuluru, O. N. Matchuk, S. G. Smirnova, N. V. Orlova, I. A. Zamulaeva, M. Garcia-Marcos, X. Li, Z. T. Young, J. N. Rauch, J. E. Gestwicki, S. Takayama and M. Y. Sherman (2014). “Hsp70-Bag3 interactions regulate cancer-related signaling networks”. Cancer research 74(17): 4731-4740.

Consolacion Melguizo, Laura Cabeza and Jose L Arias (2015): “Enhanced antitumoral activity of doxorubicin against lung cancer cells using biodegradable poly (butylcyanoacrylate) nanoparticles”. Development and therapy, DDS -92273.

Daming Zou, John Subjeck and Xiang-Yang Wang.(2016). “ Unfolding the role of large heat shock proteins: New insights and therapeutic implications” PubMed: PMC 4771732.

Daugaard, M., Jaattela, M. & Rohde, M. (2005).” Hsp70-2 is Required for tumor cell growth and survival”. Elsevier.3702-3710.

Depani, B. P., A. A. Naik and H. A. Nair (2013).” Preparation and evaluation of chitosan based thermoreversible gels for intraperitoneal delivery of 5-fluorouracil (5-FU)”. Acta Pharm 63(4): 479-491.

Davis, M. E., Z. Chen and D. M. Shin (2008). “Nanoparticle therapeutics: an emerging treatment modality for cancer”. Nat Rev Drug Discov 7(9): 771-782.

DeSantis, C. E., S. A. Fedewa, A. Goding Sauer, J. L. Kramer, R. A. Smith and A. Jemal (2016). “Breast cancer statistics (2015): Convergence of incidence rates between black and white women." CA: A Cancer Journal for Clinicians 66(1): 31-4.

Desantis, c., Siegel, r., Bandi, p. & Jemal, a. (2011).” Breast cancer statistics”, 2011. Ca cancer j clin, 61, 409-18.

Daugaard, M., M. Rohde and M. Jaattela (2007). “The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions”. FEBS Lett 581(19): 3702-3710.

De Bacco, F., P. Luraghi, E. Medico, G. Reato, F. Girolami, T. Perera, P. Gabriele, P. M. Comoglio and C. Boccaccio (2011). “Induction of MET by Ionizing Radiation and Its Role in Radioresistance and Invasive Growth of Cancer”. JNCI: Journal of the National Cancer Institute 103(8): 645-661.

De Francesco, E. M., G. Bonuccelli, M. Maggiolini, F. Sotgia and M. P. Lisanti (2017). “Vitamin C and Doxycycline: A synthetic lethal combination therapy targeting metabolic flexibility in cancer stem cells (CSCs)”. Oncotarget 8(40): 67269-67286.

Demidenko, Z. N., C. Vivo, H. D. Halicka, C. J. Li, K. Bhalla, E. V. Broude and M. V. Blagosklonny (2005). “Pharmacological induction of Hsp70 protects apoptosis-prone cells from

doxorubicin: comparison with caspase-inhibitor- and cycle-arrest-mediated cytoprotection". Cell Death and Differentiation 13: 1434.

Dent, P., A. Yacoub, P. B. Fisher, M. P. Hagan and S. Grant (2003)." MAPK pathways in radiation responses". Oncogene 22: 5885.

Dorudi, S., Steele, R. J. C. & Mcardle, C. S. (2002). "Surgery for colorectal cancer". British Medical Bulletin, 64, 101-118.

Driton, V., Luca, C., Giulia, B., Marco, C., Giovanni Filippo, P. & Lisbeth, I. (2013). "Folic Acid Conjugated Chitosan Nanoparticles for Tumor Targeting of Therapeutic and Imaging Agents"". Pharmaceutical Nanotechnology, 1, 184-203.

Elengoe, A., M. A. Naser and S. Hamdan (2015). "A Novel Protein Interaction between Nucleotide Binding Domain of Hsp70 and p53 Motif". International Journal of Genomics 2015: 391293.

Enlund, E., S. Fischer, R. Handrick, K. Otte, K.-M. Debatin, M. Wabitsch and P. Fischer-Posovszky (2014). "Establishment of Lipofection for Studying MiRNA Function in Human Adipocytes". PLoS ONE 9(5): e98023.

Fang, X., Y. Jiang, L. Feng, H. Chen, C. Zhen, M. Ding and X. Wang (2013). "Blockade of PI3K/AKT pathway enhances sensitivity of Raji cells to chemotherapy through down-regulation of HSP70". Cancer Cell International 13: 48-48.

Fink, S. L. and B. T. Cookson (2005). "Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells." Infection and Immunity 73(4): 1907-1916.

Fischer, M., M. Quaas, A. Nickel and K. Engeland (2015). "Indirect p53-dependent transcriptional repression of Survivin, CDC25C, and PLK1 genes requires the cyclin-dependent kinase inhibitor p21/CDKN1A and CDE/CHR promoter sites binding the DREAM complex." Oncotarget 6(39): 41402-41417.

Focaccetti, C., A. Bruno, E. Magnani, D. Bartolini, E. Principi, K. Dallaglio, E. O. Bucci, G. Finzi, F. Sessa, D. M. Noonan and A. Albini (2015). "Effects of 5-fluorouracil on morphology, cell cycle, proliferation, apoptosis, autophagy and ROS production in endothelial cells and cardiomyocytes." PLoS One 10(2): e0115686.

Fricker, M., J. O'Prey, A. M. Tolkovsky and K. M. Ryan (2010). "Phosphorylation of Puma modulates its apoptotic function by regulating protein stability." Cell Death Dis 1: e59.

Gao, N., Budhraja, A., Cheng, S., Yao, H., Zhang, Z. & Shi, X. 2009." Induction of Apoptosis in Human Leukemia Cells by Grape Seed Extract Occurs via Activation of c-Jun NH₂-Terminal Kinase". Clinical Cancer Research, 15, 140-149.

Gartel, A. L. & Tyner, A. L. 2002. "The role of the cyclin-dependent kinase inhibitor p21 in apoptosis". Mol Cancer Ther, 1, 639-49.

Gadaleta-Caldarola, G., S. Infusino, I. Galise, G. Ranieri, G. Vinciarelli, V. Fazio, R. Divella, A. Daniele, G. Filippelli and C. D. Gadaleta (2014). "Sorafenib and locoregional deep electro-hyperthermia in advanced hepatocellular carcinoma: A phase II study." Oncol Lett 8(4): 1783-1787.

Gao, S., E. Seker, M. Casali, F. Wang, S. S. Bale, G. M. Price and M. L. Yarmush (2012). "Ex Vivo Gene Delivery to Hepatocytes: Techniques, Challenges, and Underlying Mechanisms." Annals of biomedical engineering 40(9): 1851-1861.

Goldstein, I., V. Marcel, M. Olivier, M. Oren, V. Rotter and P. Hainaut (2011). "Understanding wild-type and mutant p53 activities in human cancer: new landmarks on the way to targeted therapies." Cancer Gene Ther 18(1): 2-11.

Guo, F., C. Sigua, P. Bali, P. George, W. Fiskus, A. Scuto, S. Annavarapu, A. Mouttaki, G. Sondarva, S. Wei, J. Wu, J. Djeu and K. Bhalla (2005). "Mechanistic role of heat shock protein 70 in Bcr-Abl-mediated resistance to apoptosis in human acute leukemia cells." Blood 105(3): 1246-1255.

Grivicich, I., A. Regner, C. Zanoni, L. P. Correa, G. P. Jotz, J. A. P. Henriques, G. Schwartsmann and A. B. da Rocha (2007). "Hsp70 response to 5-fluorouracil treatment in human colon cancer cell lines." International Journal of Colorectal Disease 22(10): 1201-1208.

Goloudina, A. R., O. N. Demidov and C. Garrido "Inhibition of HSP70: A challenging anti-cancer strategy." Cancer Letters 325(2): 117-124.

Ghasemi, Z., R. Dinarvand, F. Mottaghitalab, M. Esfandyari-Manesh, E. Sayari and F. Atyabi (2015). "Aptamer decorated hyaluronan/chitosan nanoparticles for targeted delivery of 5-fluorouracil to MUC1 overexpressing adenocarcinomas." Carbohydrate Polymers 121: 190-198.

Goliaei B;(1998) Effects of hyperthermia and granulocyte macrophage colony stimulating factor on the differentiation of human leukemic cell line U937; Pubmed: 9680097.

Galanos, P., K. Vougas, D. Walter, A. Polyzos, A. Maya-Mendoza, E. J. Haagensen, A. Kokkalis, F.-M. Roumelioti, S. Gagos, M. Tzetis, B. Canovas, A. Igea, A. K. Ahuja, R. Zellweger, S. Havaki, E. Kanavakis, D. Kletsas, I. B. Roninson, S. D. Garbis, M. Lopes, A. Nebreda, D. Thanos, J. J. Blow, P. Townsend, C. S. Sorensen, J. Bartek and V. G. Gorgoulis (2016). "Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing." Nat Cell Biol 18(7): 777-789.

Giaccone, G. (2004). "The Role of Gefitinib in Lung Cancer Treatment." Clinical Cancer Research 10(12): 4233s-4237s.

Giustini, A. J., Petryk, A. A., Cassim, S. M., Tate, J. A., Baker, I. & Hoopes, P. J. 2010.” Magnetic Nanoparticle Hyperthermia in Cancer Treatment”. Nano Life, 1, 10.1142/S179.

Guha, P., Dey, A., Sen, R., Chatterjee, M., Chattopadhyay, S. & Bandyopadhyay, S. K. 2011. Intracellular GSH Depletion Triggered Mitochondrial Bax Translocation to Accomplish Resveratrol-Induced Apoptosis in the U937 Cell Line. *Journal of Pharmacology and Experimental Therapeutics,* 336, 206-214.

Hartmann, L. C., T. A. Sellers, M. H. Frost, W. L. Lingle, A. C. Degnim, K. Ghosh, R. A. Vierkant, S. D. Maloney, V. S. Pankratz, D. W. Hillman, V. J. Suman, J. Johnson, C. Blake, T. Tlsty, C. M. Vachon, L. J. I. Melton and D. W. Visscher (2005). "Benign Breast Disease and the Risk of Breast Cancer." New England Journal of Medicine 353(3): 229-237.

Hanahan, D. and J. Folkman (1996). "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis." Cell 86(3): 353-364.
designs; int J Nanomedicine; 6:1117

Haddad-Mashadrizeh, A., Matin, M., Bahrami, A. R., Edalatmanesh, M. A., Naderi-Meshkin, H., Mousavi, S. & Gardaneh, M. (2013). “cytotoxicity and biocompatibility evaluation of chitosan-beta glycerol phosphate-hydroxyethyl cellulose hydrogel on adult rat liver for cell-based therapeutic applications”. international journal of biomedical engineering and technology, 12, 228-239.

Haupt, s., Berger, M., Goldberg, Z. & Haupt, y. (2003). “Apoptosis - the p53 network”. journal of cell science, 116, 4077-4085.

Hejazi, R. and M. Amiji (2003). "Chitosan-based gastrointestinal delivery systems." J Control Release 89(2): 151-165.

Hein, A. L., M. M. Ouellette and Y. Yan (2014). "Radiation-induced signaling pathways that promote cancer cell survival (Review)." International Journal of Oncology 45(5): 1813-1819.

Hildebrandt, B., P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix and H. Riess (2002). "The cellular and molecular basis of hyperthermia." Crit Rev Oncol Hematol 43(1): 33-56.

Hou, J.-Y., L.-N. Gao, F.-Y. Meng and Y.-L. Cui (2014) "Mucoadhesive microparticles for gastroretentive delivery: preparation, bio distribution and targeting evaluation." Marine drugs 12, 5764-5787 DOI: 10.3390/md12125764.

Horowitz, M. and S. D. M. Robinson (2007). "Heat shock proteins and the heat shock response during hyperthermia and its modulation by altered physiological conditions." Progress in Brain Research 162: 433-446

Huang, D. C., J. M. Adams and S. Cory (1998). "The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4." EMBO J 17(4): 1029-1039.

Huang, Y., Qiu, X. & Chen, J.-L. (2010). “Identification of cancer stem cells: from leukemia to solid cancers”. Frontiers in Biology, 5, 407-416.

Hwang, T. S., H. S. Han, H. K. Choi, Y. J. Lee, Y. J. Kim, M. Y. Han and Y. M. Park (2003). "Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer." J Gastroenterol Hepatol 18(6): 690-700.

Jagadish, N., S. Agarwal, N. Gupta, R. Fatima, S. Devi, V. Kumar, V. Suri, R. Kumar, V. Suri, T. C. Sadasukhi, A. Gupta, A. S. Ansari, N. K. Lohiya and A. Suri (2016). "Heat shock protein 70-2 (HSP70-2) overexpression in breast cancer." Journal of Experimental & Clinical Cancer Research 35(1): 150.

Jafar Ai, Esmaeil Biazar,Hadi G H Rad; (2011) "Nanotoxicology and nanoparticle safety in biomedical". Dove press Journal. 1117-1127

Jian Li, Sia Lee Yoong and Giorgia Pastorin;(2015) "Controlled release of cisplatin from gold Nano bottles via cleavable linkages" international journal nanomedicine: vol-10;7425-7441.

Jagadish, N., D. Parashar, N. Gupta, S. Agarwal, V. Suri, R. Kumar, V. Suri, T. C. Sadasukhi, A. Gupta, A. S. Ansari, N. K. Lohiya and A. Suri (2016). "Heat shock protein 70–2 (HSP70-2) is a novel therapeutic target for colorectal cancer and is associated with tumor growth." BMC Cancer 16(1): 561.

Jeong, J. H. & Chang, Y. C. (2010). "Ascochlorin, An Isoprenoid Antibiotic, Induces G1 Arrest Via Downregulation Of C-Myc In a P53-Independent Manner". Biochem Biophys Res Commun, 398, 68-73.

Jeong, J. H., Kang, S. S., Park, K. K., Chang, H. W., Magae, J. & Chang, Y. C. (2010). "P53-Independent Induction of G1 Arrest and P21waf1/Cip1 Expression by Ascofuranone, An Isoprenoid Antibiotic, Through Downregulation Of C-Myc". Mol Cancer Ther, 9, 2102-13.

Jinturkar, K. A. & Misra, A. (2011). "2 - Challenges and Opportunities in Gene Delivery. Challenges in Delivery of Therapeutic Genomics and Proteomics". London: Elsevier.

Jolly, C. & Morimoto, R. I. (2000). " Role of The Heat Shock Response and Molecular Chaperones in Oncogenesis and Cell Death". Jnci: Journal of The National Cancer Institute, 92, 1564-1572.

Jolesch, A., K. Elmer, H. Bendz, R. D. Issels and E. Noessner (2012). "Hsp70, a messenger from hyperthermia for the immune system." European Journal of Cell Biology 91(1): 48-52.

Jones, A. M., I. M. Westwood, J. D. Osborne, T. P. Matthews, M. D. Cheeseman, M. G. Rowlands, F. Jeganathan, R. Burke, D. Lee, N. Kadi, M. Liu, M. Richards, C. McAndrew, N. Yahya, S. E. Dobson, K. Jones, P. Workman, I. Collins and R. L. M. van Montfort (2016). "A fragment-based approach applied to a highly flexible target: Insights and challenges towards the inhibition of HSP70 isoforms." Scientific Reports 6: 34701.

Jung A Ko;(2014);" Chitosan microparticle preparation for controlled drug release by response surface methodology"; pubmed: 14594667.

Kabakov, A. E., Y. V. Malyutina and D. S. Latchman (2006). "Hsf1-mediated stress response can transiently enhance cellular radioresistance." Radiat Res 165(4): 410-423.

Kamińska, M., Ciszewski, T., Topacka-szatan, k., Miotła, P. & Starosławska, e. (2015). “Breast cancer risk factors”. menopause review, 14, 196-202.

Kaul, G. & Thippeswamy, H. (2011). “Role of heat shock proteins in diseases and their therapeutic potential”. Indian journal of microbiology, 51, 124-131.

Katas, H., M. A. G. Raja and K. L. Lam (2013). "Development of Chitosan Nanoparticles as a Stable Drug Delivery System for Protein/siRNA." International Journal of Biomaterials: 9.

Kaiser M, Kuhl A, Reins J, Fischer S;(2011); “Antileukemic activity of the HSP70 inhibitor Pifithrin-μ in acute leukemia”: pubmed: 22829184.

Kalpana Nagpal;(2010); “Chitosan nanoparticles: A promising system in novel drug delivery”; Pubmed:21048331.

Keller, D., X. Zeng, X. Li, M. Kapoor, M. S. Iordanov, Y. Taya, G. Lozano, B. Magun and H. Lu (1999). "The p38MAPK inhibitor SB203580 alleviates ultraviolet-induced phosphorylation at serine 389 but not serine 15 and activation of p53." Biochem Biophys Res Commun 261(2): 464-471.

Kevin Affram, Ofonime Udofot and Edward Agyare;(2015). “Invitro and in vivo antitumor activity of gemcitabine loaded thermosensitive liposomal nanoparticles and mild hyperthermia in pancreatic cancer”:Pubmed:26677454.

Kilkkinen, A., H. Rissanen, T. Klaukka, E. Pukkala, M. Heliovaara, P. Huovinen, S. Mannisto, A. Aromaa and P. Knekt (2008). "Antibiotic use predicts an increased risk of cancer." Int J Cancer 123(9): 2152-2155.

Klink, M., M. Nowak, M. Kielbik, K. Bednarska, E. Blus, M. Szpakowski, K. Szyllo and Z. Sulowska (2012). "The interaction of HspA1A with TLR2 and TLR4 in the response of neutrophils induced by ovarian cancer cells in vitro." Cell Stress Chaperones 17(6): 661-674.

Kuhlmann, I. (1995). "The prophylactic use of antibiotics in cell culture." Cytotechnology 19(2): 95-105.

Lai, D., Visser-Grieve, S. & Yang, X. (2012). “Tumour suppressor genes in chemotherapeutic drug response”. Bioscience Reports, 32, 361-374.

Lee, S. H., Park, S. M., Park, S. M., Park, J. H., Shin, D. Y., Kim, G. Y., Ryu, C. H., Shin, S. C., Jung, J. M., Kang, H. S., Lee, W. S. & Choi, Y. H. (2009). “Induction of apoptosis in human leukemia U937 cells by anthocyanins through down-regulation of Bcl-2 and activation of caspases”. Int J Oncol, 34, 1077-83.

L., S. R., D., M. K. & Ahmedin J. (2018).” Cancer statistics, 2018”. CA: A Cancer Journal for Clinicians, 68, 7-30.

Lee, S. Y., E. K. Jeong, M. K. Ju, H. M. Jeon, M. Y. Kim, C. H. Kim, H. G. Park, S. I. Han and H. S. Kang (2017). "Induction of metastasis, cancer stem cell phenotype, and oncogenic metabolism in cancer cells by ionizing radiation." Molecular Cancer 16: 10.

Lebret, T., R. W. G. Watson, V. Molinié, A. O'Neill, C. Gabriel, J. M. Fitzpatrick and H. Botto (2003). "Heat shock proteins HSP27, HSP60, HSP70, and HSP90." Cancer 98(5): 970-977.

Liu, Z., K. Chen, C. Davis, S. Sherlock, Q. Cao, X. Chen and H. Dai (2008). "Drug delivery with carbon nanotubes for in vivo cancer treatment." Cancer Res 68(16): 6652-6660.

Li, Z., Zhang, L. & Li, Q. (2015). “Induction of Apoptosis in Cancer Cells Through N-Acetyl-L-Leucine-Modified Polyethylenimine-Mediated P53 Gene Delivery”. Colloids and Surfaces B: Biointerfaces, 135, 630-638.

Li, Y., Dowbenko, D. & Lasky, L. A. (2002). “AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival”. J Biol Chem, 277, 11352-61.

Li, X., T. Colvin, J. N. Rauch, D. Acosta-Alvear, M. Kampmann, B. Dunyak, B. Hann, B. T. Aftab, M. Murnane, M. Cho, P. Walter, J. S. Weissman, M. Y. Sherman and J. E. Gestwicki (2015). "Validation of the Hsp70-Bag3 Protein-Protein Interaction as a Potential Therapeutic Target in Cancer." Molecular cancer therapeutics 14(3): 642-648.

Llorca Ferrandiz, C., G. Esquerdo Galiana, J. M. Cervera Grau, H. C. Briceno Garcia, J. V. Calduch Broseta and J. Del Pino Cuadrado (2005). "[5-Fluorouracil-induced small bowel toxicity in a patient with colorectal cancer]." Clin Transl Oncol 7(8): 356-357.

Llobet, L., J. Montoya, E. Lopez-Gallardo and E. Ruiz-Pesini (2015). "Side Effects of Culture Media Antibiotics on Cell Differentiation." Tissue Eng Part C Methods 21(11): 1143-1147.

Luo, H., L. Cowen, G. Yu, W. Jiang and Y. Tang (2016). "SMG7 is a critical regulator of p53 stability and function in DNA damage stress response." Cell Discovery 2: 15042.

Luo, Y., Liu, Y., Hou, K., Wang, S. & Wang, Y. (2005). “Molecular mechanism of differentiation and apoptosis of U937 cells induced by 12-O-tetradecanoylphorbol-13-acetate”. Chinese Journal of Clinical Oncology, 2, 553-557.

Maria Jose Alonso and Alejandro Sanchez:2003.The potential of chitosan in ocular drug delivery; Journal of pharmacy and pharmacology; 55-1451 – 1463.

Maddams, J., M. Utley and H. Møller (2012). "Projections of cancer prevalence in the United Kingdom, 2010–2040." British Journal of Cancer 107: 1195.

Marchand, C., G. E. Rivard, J. Sun and C. D. Hoemann (2009). "Solidification mechanisms of chitosan–glycerol phosphate/blood implant for articular cartilage repair." Osteoarthritis and Cartilage 17(7): 953-960.

Mahattanadul, N., P. Sunintaboon, P. Sirithip and P. Tuchinda (2016). "Chitosan-functionalised poly (2-hydroxyethyl methacrylate) core-shell microgels as drug delivery carriers: salicylic acid loading and release." Journal of Microencapsulation 33(6): 563-568.

Majid, S., Kikuno, N., Nelles, J., Noonan, E., Tanaka, Y., Kawamoto, K., Hirata, H., Li, L. C., Zhao, H., Okino, S. T., Place, R. F., Pookot, D. & Dahiya, R. (2008). "Genistein Induces Tumor Suppressor Genes in Prostate Cancer Cells by Epigenetic Mechanisms Involving Active Chromatin Modification". Cancer Research, 68, 2736-2744.

Maier, P., L. Hartmann, F. Wenz and C. Herskind (2016). "Cellular Pathways in Response to Ionizing Radiation and Their Targetability for Tumor Radiosensitization." International Journal of Molecular Sciences 17(1): 102.

Manuel Banobre -lopez, Antonio Teijeiro, Jose Rivas;(2013); "Magnetic nanoparticle based hyperthermia for cancer treatment"; science direct; vol-18L6);397-400.

Mansilla, M. J., C. Costa, H. Eixarch, V. Tepavcevic, M. Castillo, R. Martin, C. Lubetzki, M.-S. Aigrot, X. Montalban and C. Espejo (2014). "Hsp70 Regulates Immune Response in Experimental Autoimmune Encephalomyelitis." PLoS ONE 9(8): e105737.

Mahmood M, Karmakar A, Fejleh A, Mocan T,Iancu.C ,Mocan L ,Lancu D T,Xu Y, Dervishi E,LiZ ,Biris AR, Agarwal R, Ali N,Galanzha EI, Biris AS, Zharov VP:(2009) "Synergistic enhancement of cancer therapy using a combination of carbon nanotubes and anti-tumor drug:Nanomedicine" (Lond);883-93.(Pubmed).

McCallister, C., B. Kdeiss and N. Nikolaidis (2015). "HspA1A, a 70-kDa heat shock protein, differentially interacts with anionic lipids." Biochemical and Biophysical Research Communications 467(4): 835-840.

Mckeon, A., Egan, A., Chandanshive, J., Mcmahon, H. & Griffith, D. (2016). "Novel Improved Synthesis of HSP70 Inhibitor, Pifithrin-μ. In Vitro Synergy Quantification of Pifithrin-μ Combined with Pt Drugs in Prostate and Colorectal Cancer Cells". *Molecules,* 21, 949.

Mizushima, N., B. Levine, A. M. Cuervo and D. J. Klionsky (2008). "Autophagy fights disease through cellular self-digestion." Nature 451(7182): 1069-1075.

Milani V, Noessner E, Ghose S, Kuppner M, Ahrens B(2002); "Heat shock protein 70: role in antigen presentation and immune stimulation"; Pubmed: 12537755.

Murphy, M. E. (2013). "The HSP70 family and cancer." Carcinogenesis 34(6): 1181-1188.

Multhoff, G. (2006). "Heat shock proteins in immunity." Handb Exp Pharmacol (172): 279-304.

Müller, R. H., Mäder, K. & Gohla, S. (2000).” Solid Lipid Nanoparticles (Sln) For Controlled Drug Delivery – A Review of The State of The Art”. European Journal of Pharmaceutics and Biopharmaceutics, 50, 161-177.

Murakami, N., A. Kuhnel, T. E. Schmid, K. Ilicic, S. Stangl, I. S. Braun, M. Gehrmann, M. Molls, J. Itami and G. Multhoff (2015). "Role of membrane Hsp70 in radiation sensitivity of tumor cells." Radiat Oncol 10: 149.

Mucsi, Z., Chass, G. A., Abranyi-Balogh, P., Jojart, B., Fang, D.-C., Ramirez-Cuesta, A. J., Viskolcz, B. & Csizmadia, I. G. (2013). “Penicillin's catalytic mechanism revealed by inelastic neutrons and quantum chemical theory”. Physical Chemistry Chemical Physics, 15, 20447-20455.

Modi R Sheetal, James.A. Collin, David A, Relmann (2014);” Antibiotics and the gut microbiota”; The journal of clinical investigation; 10. 1172. Jc172333.

Modi, S. R., J. J. Collins and D. A. Relman (2014). "Antibiotics and the gut microbiota." The Journal of Clinical Investigation 124(10): 4212-4218.

Nam, J.-P., Choi, C., Hang, M.-K., Jeong, Y.-I., Nah, J.-W., Kim, S.-H. & Park, Y. (2010).” Insulin-Incorporated Chitosan Nanoparticles Based on Polyelectrolyte Complex Formation”. Macromolecular Research, 18, 630-635.

Nakamura H, Fang J, (2013); “The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo”; Pubmed: 23088862.

Niidome T, Huang L(2002); “Gene therapy progress and prospects: nonviral vectors”; pubmed:12457277.

Oei, A. L., L. E. M. Vriend, J. Crezee, N. A. P. Franken and P. M. Krawczyk (2015). "Effects of hyperthermia on DNA repair pathways: one treatment to inhibit them all." Radiation Oncology (London, England) 10: 165.

Ohigashi, S., Sudo, K., Kobayashi, D., Takahashi, O., Takahashi, T., Asahara, T., Nomoto, K. & Onodera, H. (2013).” Occurence of initiation and progression of colorectal cancer in the changed intestinal environment”. Journal of Clinical Oncology, 31, 416-416.

Park, K.-S., Ahn, Y., Kim, J.-A., Yun, M.-S., Seong, B. L. & Choi, K.-Y. 2002. Extracellular zinc stimulates ERK-dependent activation of p21Cip/WAF1 and inhibits proliferation of colorectal cancer cells. British Journal of Pharmacology, 137, 597-607.

Padua, D. and J. Massague (2009). "Roles of TGFbeta in metastasis." Cell Res 19(1): 89-102.
Patel, M. P., R. R. Patel and J. K. Patel (2010). "Chitosan mediated targeted drug delivery system: a review." J Pharm Sci 13(4): 536-557.

Pasz-Walczak, G., R. Kordek and M. Faflik (2001). "P21 (WAF1) Expression in Colorectal Cancer: Correlation with P53 and Cyclin D1 Expression, Clinicopathological Parameters and Prognosis." Pathology - Research and Practice 197(10): 683-689.

Pavelic, S. K., T. Cacev and M. Kralj (2008). "A dual role of p21waf1/cip1 gene in apoptosis of HEp-2 treated with cisplatin or methotrexate." Cancer Gene Ther 15(9): 576-590.

Ploner, C., R. Kofler and A. Villunger (2008). "Noxa: at the tip of the balance between life and death." Oncogene 27(Suppl 1): S84-S92.

Parrales, A. and T. Iwakuma (2015). "Targeting Oncogenic Mutant p53 for Cancer Therapy." Frontiers in Oncology 5: 288.

Pereira, L. P., P. Silva, M. Duarte, L. Rodrigues, C. M. M. Duarte, C. Albuquerque and A. T. Serra (2017). "Targeting Colorectal Cancer Proliferation, Stemness and Metastatic Potential Using Brassicaceae Extracts Enriched in Isothiocyanates: A 3D Cell Model-Based Study." Nutrients 9(4): 368.

Pearson S, Hepeng Jia and Keiko Kandachi (2004); “China approves first gene therapy”; Nature Biotechnology;22;3-4.

Puga, A. M., A. C. Lima, J. F. Mano, A. Concheiro and C. Alvarez-Lorenzo (2013). "Pectin-coated chitosan microgels crosslinked on superhydrophobic surfaces for 5-fluorouracil encapsulation." Carbohydrate Polymers 98(1): 331-340.

Qi, L. and Z. Xu (2006). "In vivo antitumor activity of chitosan nanoparticles." Bioorganic & Medicinal Chemistry Letters 16(16): 4243-4245.

Quintana. C., Cabrera, J., Perdomo, J., Estevez, F., Lora, J. F., Reiter, R. J. & Quintana, J. (2016). “Melatonin enhances hyperthermia-induced apoptotic cell death in human leukemia cells”. j pineal res, 61, 381-95.

Ravizza, R., M. B. Gariboldi, L. Passarelli and E. Monti (2004). "Role of the p53/p21 system in the response of human colon carcinoma cells to Doxorubicin." BMC Cancer 4(1): 92.

Rau, B., I. Sturm, H. Lage, S. Berger, U. Schneider, S. Hauptmann, P. Wust, H. Riess, P. M. Schlag, B. Dörken and P. T. Daniel (2003). "Dynamic Expression Profile of p21WAF1/CIP1 and Ki-67 Predicts Survival in Rectal Carcinoma Treated with Preoperative Radio chemotherapy." Journal of Clinical Oncology 21(18): 3391-3401.

Rao, V. A., S. R. Klein, K. K. Agama, E. Toyoda, N. Adachi, Y. Pommier and E. B. Shacter (2009). "The iron chelator Dp44mT causes DNA damage and selective inhibition of topoisomerase IIalpha in breast cancer cells." Cancer Res 69(3): 948-957.

Rao, V. A., J. Zhang, S. R. Klein, P. Espandiari, A. Knapton, J. S. Dickey, E. Herman and E. B. Shacter (2011). "The iron chelator Dp44mT inhibits the proliferation of cancer cells but fails to protect from doxorubicin-induced cardiotoxicity in spontaneously hypertensive rats." Cancer Chemotherapy and Pharmacology 68(5): 1125-1134.

Reuter, S., S. C. Gupta, M. M. Chaturvedi and B. B. Aggarwal (2010). "Oxidative stress, inflammation, and cancer: How are they linked?" Free radical biology & medicine 49(11): 1603-1616.

Ritossa, F. (1996). "Discovery of the heat shock response." Cell Stress & Chaperones 1(2): 97-98.

Richard G, Shengnan YU, Anping Li, Xun, Yuan; (2017); " Recent advances of bispecific antibodies in solid tumors"; Pubmed 13045-017-0522-z.

Richard P Junghans (2017)"The challenges of solid tumor for designer CAR-T therapies: a 25-year perspective' 'Cancer Gene Therapy24,89-99.

Rothenberg, M. L. (2001). "Irinotecan (CPT-11): Recent Developments and Future Directions–Colorectal Cancer and Beyond." The Oncologist 6(1): 66-80.

Rohde, M., M. Daugaard, M. H. Jensen, K. Helin, J. Nylandsted and M. Jäättelä (2005). "Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms." Genes & Development 19(5): 570-582.

Rundhaug, J. E. (2003). "Matrix Metalloproteinases, Angiogenesis, and Cancer. Reduction of Wound Angiogenesis in Patients Treated with BMS-275291, a Broad-Spectrum Matrix Metalloproteinase Inhibitor. Clin. Cancer Res. 9(2): 551-554.

Ryu, A. H., Eckalbar, W. L., Kreimer, A., Yosef, N. & Ahituv, N. (2017);" Use antibiotics in cell culture with caution: genome-wide identification of antibiotic-induced changes in gene expression and regulation". Scientific Reports, 7, 7533.

Salucci, S., S. Burattini, M. Battistelli, V. Baldassarri, M. C. Maltarello and E. Falcieri (2013). "Ultraviolet B (UVB) Irradiation-Induced Apoptosis in Various Cell Lineages in Vitro." International Journal of Molecular Sciences 14(1): 532-546.

Sandu, c., Ngounou wetie, A.G., Darie, C.C. & Steller, h. (2014). "Thiostrepton, a natural compound that triggers heat shock response and apoptosis in human cancer cells: a proteomics investigation". adv Exp med Biol, 806, 443-51.

Sanchaika Guar, Yunfei Wen, Jian H. Song, Nila U. Parikh, LingegowdaS. Mangala, Alicia M. Blessing, Cristina Ivan, Sherry Y. Wu, Andreas Varkaris, Yan Shi, Gabriel Lopez- Berestein, Saranya, N., A. Moorthi, S. Saravanan, M. P. Devi and N. Selvamurugan (2011). "Chitosan and its derivatives for gene delivery." Int J Biol Macromol 48(2): 234-238.

Saha, C., A. Kaushik, A. Das, S. Pal and D. Majumder (2016). "Anthracycline Drugs on Modified Surface of Quercetin-Loaded Polymer Nanoparticles: A Dual Drug Delivery Model for Cancer Treatment." PLoS One 11(5): e0155710.

Schmitt, C. A., J. S. Fridman, M. Yang, S. Lee, E. Baranov, R. M. Hoffman and S. W. Lowe (2002). "A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy." Cell 109(3): 335-346.

Schlörmann, W., S. Naumann, C. Renner and M. Glei (2015). "Influence of miRNA-106b and miRNA-135a on butyrate-regulated expression of p21 and Cyclin D2 in human colon adenoma cells." Genes & Nutrition 10(6): 50.

Schuurbiers, O., Kaanders, J., Van Der Heijden, E. H. F. M., P.N. Dekhuijzen, R., Oyen, W. & Bussink, J. (2009). "The PI3-K/AKT-Pathway and Radiation Resistance Mechanisms in Non-small Cell Lung Cancer"J.oncology S-1556.

Schildkopf P, Frey B, Ott O J, Rubner, Multhoff G, Sauer R, Fietkau R, Gaipi US;(2011); "Radiation combined with hyperthermia induces HSP70- dependent maturation of dentric cells and release of pro-inflammatory cytokines by dentric cells and macrophages"; pubmed: 21716.

Sekihara, K., N. Harashima, M. Tongu, Y. Tamaki, N. Uchida, T. Inomata and M. Harada (2013). "Pifithrin-mu, an inhibitor of heat-shock protein 70, can increase the antitumor effects of hyperthermia against human prostate cancer cells." PLoS One 8(11): e78772.

Sherman, M. Y. and V. L. Gabai (2015). "Hsp70 in cancer: back to the future." Oncogene 34(32): 4153-4161.

Shibue, T., K. Takeda, E. Oda, H. Tanaka, H. Murasawa, A. Takaoka, Y. Morishita, S. Akira, T. Taniguchi and N. Tanaka (2003). "Integral role of Noxa in p53-mediated apoptotic response." Genes & Development 17(18): 2233-2238.

Shakibaei, M., P. Kraehe, B. Popper, P. Shayan, A. Goel and C. Buhrmann (2015). "Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer." BMC Cancer 15: 250.

Shao, Z. M., Z. Z. Shen, C. H. Liu, M. R. Sartippour, V. L. Go, D. Heber and M. Nguyen (2002). "Curcumin exerts multiple suppressive effects on human breast carcinoma cells." Int J Cancer 98(2): 234-240.

Shafie, N. H., Esa, N. M., Ithnin, H., Saad, N. & Pandurangan, A. K. (2013). "Pro-Apoptotic effect of rice bran inositol hexaphosphate (IP6) on HT-29 colorectal cancer cells". Int J Mol Sci, 14, 23545-58.

Siegel, R., C. Desantis and A. Jemal (2014). "Colorectal cancer statistics, 2014." CA Cancer J Clin 64(2): 104-117.

Siegel, r. L., Miller, k. D. & Jemal, a. (2017). "Cancer statistics, 2017". cancer journal for clinicians, 67, 7-30.

Skitzki, J. J., E. A. Repasky and S. S. Evans (2009). "Hyperthermia as an immunotherapy strategy for cancer." Current opinion in investigational drugs (London, England: 2000) 10(6): 550-558.

Son, K.-j., K. r. Choi, S. J. Lee and H. Lee (2016). "Immunogenic Cell Death Induced by Ginsenoside Rg3: Significance in Dendritic Cell-based Anti-Tumor Immunotherapy." Immune Network 16(1): 75-84.

Sork, H., J. Z. Nordin, J. J. Turunen, O. P. B. Wiklander, B. Bestas, E. M. Zaghloul, H. Margus, K. Padari, A. D. Duru, G. Corso, J. Bost, P. Vader, M. Pooga, C. I. E. Smith, M. J. A. Wood, R. M. Schiffelers, M. Hällbrink and S. E. L. Andaloussi (2016). "Lipid-based Transfection Reagents Exhibit Cryo-induced Increase in Transfection Efficiency." Molecular Therapy. Nucleic Acids 5(3): e290.

Solowey, E., M. Lichtenstein, S. Sallon, H. Paavilainen, E. Solowey and H. Lorberboum-Galski (2014). "Evaluating Medicinal Plants for Anticancer Activity." The Scientific World Journal 2014: 721402.

Seo, J. H., Park, J. H., Lee, E. J., Vo, T. T., Choi, H., Kim, J. Y., Jang, J. K., Wee, H. J., Lee, H. S., Jang, S. H., Park, Z. Y., Jeong, J., Lee, K. J., Seok, S. H., Park, J. Y., Lee, B. J., Lee, M. N., Oh, G. T. & Kim, K. W. (2016); "Ard1-Mediated Hsp70 acetylation balances stress-induced protein refolding and degradation". Nat Commun, 7, 12882.

Song, X., Kim, S. Y., Zhou, Z., Lagasse, E., Kwon, Y. T. & Lee, Y. J. (2013). "Hyperthermia enhances mapatumumab-induced apoptotic death through ubiquitin-mediated degradation of cellular FLIP (long) in human colon cancer cells". Cell Death & Disease, 4, e577.

Song, G., Mao, Y. B., Cai, Q. F., Yao, L. M., Ouyang, G. L. & Bao, S. D. (2005). "Curcumin induces human HT-29 colon adenocarcinoma cell apoptosis by activating p53 and regulating apoptosis-related protein expression". Brazilian Journal of Medical and Biological Research, 38, 1791-1798.

Susan, E. (2007). "Apoptosis: A Review of Programmed Cell Death". Toxicologic Pathology, 35, 495-516.

S. Wimardhani, Y., D. F. Suniarti, H. J. Freisleben, S. I. Wanandi, N. C. Siregar and M.-A. Ikeda (2014). "Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells." Journal of Oral Science 56(2): 119-126.

Takeda, K., A. Wada, K. Yamamoto, Y. Moriyama and K. Aoki (1989). "Conformational change of bovine serum albumin by heat treatment." J Protein Chem 8(5): 653-659.

Tian, F., T. Fan, Y. Zhang, Y. Jiang and X. Zhang (2012). "Curcumin potentiates the antitumor effects of 5-FU in treatment of esophageal squamous carcinoma cells through downregulating the activation of NF-κB signaling pathway in vitro and in vivo." Acta Biochimica et Biophysica 44(10): 847-855.

Ubeda, C., Taur, Y., Jenq, R. R., Equinda, M. J., Son, T., Samstein, M., Viale, A., Socci, N. D., Van Den Brink, M. R. M., Kamboj, M. & Pamer, E. G. 2010. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. The Journal of Clinical Investigation, 120, 4332-4341.

Urano, M., L. Rice, R. Epstein, H. D. Suit and A. M. Chu (1983). "Effect of whole-body hyperthermia on cell survival, metastasis frequency, and host immunity in moderately and weakly immunogenic murine tumors." Cancer Res 43(3): 1039-1043.

Van der Zee, J. (2002). "Heating the patient: a promising approach?" Ann Oncol 13(8): 1173-1184.

Van Etten, B., T. L. M. ten Hagen, M. R. de Vries, G. Ambagtsheer, T. Huet and A. M. M. Eggermont (2002). "Prerequisites for effective adenovirus mediated gene therapy of colorectal liver metastases in the rat using an intracellular neutralizing antibody fragment to p21-Ras." Br J Cancer 86(3): 436-442.

Vinod, B. S., J. Antony, H. H. Nair, V. T. Puliyappadamba, M. Saikia, S. Shyam Narayanan, A. Bevin and R. John Anto (2013). "Mechanistic evaluation of the signaling events regulating curcumin-mediated chemosensitization of breast cancer cells to 5-fluorouracil." Cell Death Dis 4: e505.

Wong, R. S. (2011). “Apoptosis in Cancer: From Pathogenesis to Treatment”. Journal of Experimental & Clinical Cancer Research, 30, 87.

Walton, M. I., S. C. Wilson, I. R. Hardcastle, A. R. Mirza and P. Workman (2005). "An evaluation of the ability of pifithrin-α and -β to inhibit p53 function in two wild-type p53 human tumor cell lines." Molecular Cancer Therapeutics 4(9): 1369-1377.

Wang, J., Z.-j. Lei, Y. Guo, T. Wang, Z.-y. Qin, H.-l. Xiao, L.-l. Fan, D.-f. Chen, X.-w. Bian, J. Liu and B. Wang (2015). "miRNA-regulated delivery of lincRNA-p21 suppresses β-catenin signaling and tumorigenicity of colorectal cancer stem cells." Oncotarget 6(35): 37852-37870.

Westphal, D., G. Dewson, P. E. Czabotar and R. M. Kluck (2011). "Molecular biology of Bax and Bak activation and action." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1813(4): 521-531.

Wright, D. G., C. Marchal, K. Hoang, J. A. Ankney, S. T. Nguyen, A. W. Rushing, N. Polakowski, B. Miotto and I. Lemasson (2016). "Human T-cell leukemia virus type-1-encoded protein HBZ represses p53 function by inhibiting the acetyltransferase activity of p300/CBP and HBO1." Oncotarget 7(2): 1687-1706.

Wu, Z., T. Wang, Y. Zhang, Z. Zheng, S. Yu, S. Jing, S. Chen, H. Jiang and S. Ma (2017). "Anti-cancer effects of β-elemene with hyperthermia in lung cancer cells " ; Experimental and Therapeutic Medicine 13(6): 3153-3157.

Wu, X., T. Liu, O. Fang, W. Dong, F. Zhang, L. Leach, X. Hu and Z. Luo (2016). "MicroRNA-708-5p acts as a therapeutic agent against metastatic lung cancer " ; Oncotarget 7(3): 2417-2432.

Wu, J., Mukherjee, A., Lebman, D. A. & Fang, X. (2011);" Lysophosphatidic Acid–Induced P21 Expression Mediates the Cytostatic Response of Breast and Ovarian Cancer Cells to Tgfβ";. Molecular Cancer Research, 9, 1562-1570.

Xiao, C., S. Chen, M. Yuan, F. Ding, D. Yang, R. Wang, J. Li, R. M. Tanguay and T. Wu (2004). "Expression of the 60 kDa and 71 kDa heat shock proteins and presence of antibodies against the 71 kDa heat shock protein in pediatric patients with immune thrombocytopenic purpura."; BMC Hematology 4(1): 1.

Xia, W., Chen, J.-S., Zhou, X., Sun, P.-R., Lee, D.-F., Liao, Y., Zhou, B. P. & Hung, M.-C. (2004). "Phosphorylation/cytoplasmic localization of p21cip1/waf1/ is associated with her2strong overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients"; clinical cancer research, 10, 3815-3824.

Yan, J., J. Xiang, Y. Lin, J. Ma, J. Zhang, H. Zhang, J. Sun, N. N. Danial, J. Liu and A. Lin (2013). "Inactivation of the BH3-only Protein BAD by IKK Inhibits TNFα-induced Apoptosis Independently of NF-κB activation"; Cell 152(1-2): 304-315.

Yamamoto, H., J. W. Soh, H. Shirin, W. Q. Xing, J. T. Lim, Y. Yao, E. Slosberg, N. Tomita, I. Schieren and I. B. Weinstein (1999). "Comparative effects of overexpression of p27Kip1 and p21Cip1/Waf1 on growth and differentiation in human colon carcinoma cells."; Oncogene 18(1): 103-115.

Yang, K.-L., C.-C. Huang, M.-S. Chi, H.-C. Chiang, Y.-S. Wang, C.-C. Hsia, G. Andocs, H.-E. Wang and K.-H. Chi (2016). "In vitro comparison of conventional hyperthermia and modulated electro-hyperthermia."; Oncotarget 7(51): 84082-84092.

Yu, Z., M. Yu, Z. Zhang, G. Hong and Q. Xiong (2014). "Bovine serum albumin nanoparticles as controlled release carrier for local drug delivery to the inner ear;" Nanoscale Research Letters 9(1): 343-343.

Yu, X., X. Liang, H. Xie, S. Kumar, N. Ravinder, J. Potter, X. de Mollerat du Jeu and J. D. Chesnut (2016). "Improved delivery of Cas9 protein/gRNA complexes using lipofectamine CRISPRMAX"; Biotechnology Letters 38: 919-929.

Yijie Shi, Chang Su, Wenyu Cui, Hongdan Li, Liwei Liu, Bo Feng, Ming Liu, Rongjian Su and Liang Zhao;(2014); "Gefitinib loaded folate decorated bovine serum albumin conjugated carboxymethyl-beta-cyclodextrin nanoparticles enhance drug delivery and attenuate autophagy in folate receptor-positive cancer cells"; journal of nanotechnology,12:43.

Zhang, Q., K. Shim, K. Wright, A. Jurkevich and S. Khare (2016). "Atypical role of sprouty in p21 dependent inhibition of cell proliferation in colorectal cancer"; Molecular Carcinogenesis 55(9): 1355-1368.

Zhang, L., F. X. Gu, J. M. Chan, A. Z. Wang, R. S. Langer and O. C. Farokhzad (2008). "Nanoparticles in medicine: therapeutic applications and developments." Clin Pharmacol Ther 83(5): 761-769.

Zhang N, Li J, Jiang W ;(2010); Effective protection and controlled release of insulin by cationic beta- cyclodextrin polymers from alginate/chitosan nanoparticles; international journal pharms,393(1-2)213-219.

Zhang, Z., Hong, G. & Xiong, Q. (2014); Bovine Serum Albumin Nanoparticles As Controlled Release Carrier For Local Drug Delivery To The Inner Ear. Nanoscale Research Letters, 343343.

Zhao, Y., S. Zhang, P. Wang, S. Fu, D. Wu and A. Liu (2017). "Seleno-short-chain chitosan induces apoptosis in human non-small-cell lung cancer A549 cells through ROS-mediated mitochondrial pathway"; Cytotechnology 69(6): 851-863.

Ziv, e., John, e. M., Choudhry, s., Kho, j., Lorizio, w., Perez-stable, e. J. & Burchard, e. G. (2006). “Genetic ancestry and risk factors for breast cancer among latinas in the san francisco bay area. Cancer epidemiology, biomarkers & prevention”; American society of preventive oncology, 15, 1878-1885.